PARACRINE REGULATION OF THE RAT TESTIS:

STUDIES ON SEMINIFEROUS TUBULE-LEYDIG CELL COMMUNICATION

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CONTENTS

Except where acknowledgement is made by reference, the experiments described in this thesis were the unaided work of the author. All experiments were performed in the Medical Research Council's Unit of Reproductive Biology in Edinburgh.

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DEDICATION

This thesis is dedicated to God's glory, in thankfulness for His faithfulness in fulfilling His promises to me.

> Jeremiah Ch 29 vv 11-14: "For I know the plans I have for you," declares the LORD, "plans to prosper you and not to harm you, plans to give you hope and a future. Then you will call upon me and come and pray to me and I will listen to you. You will seek me and find me when you seek me with all your heart, I will be found by you," declares the LORD.

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ABSTRACT

Despite recent advances in our understanding of the possible factors involved in the local control of testicular function, reproductive endocrinologists have suffered from a dearth of *in vitro* techniques for the study of paracrine regulation of the testis. The aims of this study were to investigate novel ways of studying seminiferous tubule-Leydig cell interaction, with a view to establishing techniques which would permit the investigation of paracrine events within the testis. Linked to this, studies on the quantitative distribution of testosterone within the testis were undertaken.

Three separate systems for the investigation of seminiferous tubule-Leydig cell communication have been evaluated(Chapters 3-5) and one system has been identified, based on co-perifusion of seminiferous tubules with Leydig cells, which provides consistent and reproducible results(Chapter 5). The addition of isolated seminiferous tubules to Leydig cells in 'physiological' proportions significantly enhanced Leydig cell testosterone production. Furthermore, it has been shown that the degree of LH stimulation to which Leydig cells are exposed plays a role in determining their response to seminiferous tubule stimulation(Chapter 5). This perifusion system represents a significant improvement upon the *in vitro* systems currently available for the investigation of seminiferous tubule-Leydig cell interactions.

Techniques for the determination of intratesticular distribution of testosterone have, combined with histological techniques, provided insights into the role of testosterone in the regulation of spermato-

genesis and have also identified chemicals which may further advance the understanding of the role of intratubular cell associations in the maintenance of testicular function. It has been shown that, whilst testosterone is essential for the maintenance of spermatogenesis, many stages of the spermatogenic cycle are relatively resistant to complete testosterone withdrawal, following destruction of the Leydig cells(Chapter 7). Despite the removal of all Leydig cells from the testis, complete restoration of the Leydig cell population occurred within 10 weeks and this was associated with restoration of near normal testicular morphology. Furthermore, results from studies in which the intratesticular testosterone levels were reduced suggest that under these conditions, seminiferous tubule testosterone concentrations were conserved preferentially, either by the action of paracrine factors, or by the retention of testosterone within the tubules(Chapter 6).

The techniques established here will permit a number of novel approaches to the investigation of testicular paracrine regulation. For example, having established a reliable *in vitro* system for the investigation of seminiferous tubule-Leydig cell interaction, it should prove possible to use this as a 'bioassay' with which to assess both the role of putative paracrine factors and the functional integrity of the seminiferous tubules following various experimental procedures.

CHAPTER 1

LITERATURE REVIEW

1.1: Historical perspectives:

The testis has long been recognised as the organ involved in fertility in men. The Romans refused to allow the testimony of a man to stand unless he was able to prove that his testicles were intact, and it is from this practice that the word testify originates. The infertility of castrates, or eunuchs, was also recognised by early civilisations, and such individuals were in great demand for the guarding of harems etc.

It seems surprising then that it was not until the late seventeenth century that the existence of spermatozoa was discovered. Even then it was some time before it was accepted that these motile cells were in fact the male gametes and not just parasitic infections of the urogentital tract. However with the development of the simple early light microscopes, histologists and anatomists had access to an area of study not previously possible, and it was Hamen, a student working with one of the pioneers of microscopy, Leeuwenhoek, who first described spermatozoa in 1677. Despite the backing of Leeuwenhoek, Hamen's discovery was not accepted and it was not until 1841 that these cells were found to originate from the testis, and in 1865 that they were proved to be involved in fertilization.

Although the structure of the testis was described in 1668 it was many years later before the seminiferous tubule was recognised as the site of spermatogenesis. Microscopists described the fine structure of the tubules in the late 19th century, with the nomenclature of the cell types being derived from their discoverers. For example, the Sertoli cell was named for its Italian discoverer by the German scientist von Ebner(1888) and the Leydig cell was named after himself by von Leydig(1850). The advent of selective staining

techniques for the Leydig cell early in the 20th century and for the seminiferous epithelium around 1950 led to further investigations into the structure and function of these tissues. In 1903, on the basis of purely morphological evidence, the Leydig cell was postulated as the site of synthesis of many of the factors responsible for the development of male characteristics. Between the 1950's and 1960's the complex organization of the seminiferous epithelium was shown to be arranged in distinct and recognizable stages, for which a simple yet detailed classification system could be devised(Leblond & Clermont, 1952a,b).

Thus over the first 300 years of research the evidence for the involvement of the testis in fertility was mainly morphological. It is only over the last 25 years or so that techniques for the quantification of testicular hormones and the culture of testicular cells have enabled detailed investigation of the physiological and biochemical role of the testis. Investigation of the hormonal regulation of the testis indicated the dependence of the testis upon pituitary gonadotrophins as early as 1927. Regression of the seminiferous epithelium and Leydig cells followed the removal of the pituitary and renewal of spermatogenesis could be demonstrated only if pituitary extracts were given or if whole pituitaries were implanted(Hooker, 1970).

The presence of androgens was established in the late 1920's leading to the purification of androstenedione and testosterone in the 1930's. The separation of pituitary products in the 1930's into Luteinizing Hormone and Follicle Stimulating Hormone(LH & FSH) showed that research was still predominantly female orientated, but effects of LH on the interstitium and later its androgen-mediated effects on

the seminiferous tubules were also demonstrated around this time in hypophysectomized rats, and the ability of testosterone to maintain spermatogenesis qualitatively following hypophysectomy in rats was also shown(Smith, 1930). The ability of the gonadal steroids to influence the pituitary secretion of gonadotrophins was also demonstrated and the existence of a negative feedback control established. However the role of FSH upon the adult testis and the ability of the testis to regulate pituitary FSH production was not established at this time, although in 1932 an FSH regulatory hormone, inhibin, was postulated(McCullagh, 1932).

The sensitivity of the testis to heat and the dependence of fertility upon complete descent of the testis in animals with scrotal testes was also demonstrated in the between 1920-1935; animals in which the testis was either heated or prevented from remaining cool by insulation showed loss of fertility accompanied by a reduction in the endocrine activity of the testes(Moore, 1924).

Therefore prior to the 1960's the basis for the endocrine and biochemical investigation of the testis was laid, and the testis had been established as an organ of amazing structural organization and complexity, with major endocrine functions.

1.2: Testicular structure and organization:

The mammalian testis is separated into two major compartments, the avascular seminiferous tubules, which comprise over 80% of the testicular mass in most mammals, and the vascularized interstitium which comprises about 16% of the testicular mass and contains the Leydig cells, macrophages, and other minor cell components. Because of the high proportion of avascular tissue within the testis, it is perhaps not surprising that the majority of mammalian species have

large volumes of interstitial fluid or testicular lymph, which fills the interstitial spaces between the seminiferous tubules and provides the medium for communication and transport between the vasculature and the seminiferous tubules and interstitial cells. In rats, the volume of interstitial fluid and its testosterone concentration increase with age(Sharpe, 1979), and injection of hCG increases the volume of interstitial fluid and its testosterone concentration. hCG treatment also increases testicular capillary permeability(Setchell & Sharpe, 1981), and this may explain the increased interstitial fluid volume. This may contribute to the action of hCG/LH on testosterone production seen in these animals, since increased blood flow would result in increased LH exposure. The volume of interstitial fluid recovered from the rat testis has been shown to reflect the total extratubular interstitial fluid volume of the testis(Sharpe & Cooper, 1983) and to provide a means of assessing changes within the interstitial environment(Sharpe & Cooper, 1983). The volume of interstitial fluid also reflects changes in the permeability of testicular blood vessels(Setchell & Sharpe, 1981; Sharpe & Cooper, 1983).

The existence of a well documented barrier at the level of the basal tight junctions of the Sertoli cell(Setchell, 1980), obviously plays a part both in the maintenance of the intratesticular environment and in controlling the entry of peripheral hormones and the exit of hormones produced within the testis which act elsewhere, for example to modulate the release of gonadotrophins. However, although this is often assumed to be the only barrier within the testis there is evidence that, at two other levels of the testis, barriers exist which exclude substances from the testis. The first of these is at

the level of the testicular capiliaries, and appears during puberty as shown by Kormano(1967). The barrier excludes certain dyes from the interstitium after puberty, which previous to the formation of this barrier have free access to the testis(Kormano, 1967). Substances which cross the capillary barrier may then be excluded from entering the seminiferous tubules by either the layer of myoid cells which surround the seminiferous tubules(Dym & Fawcett, 1970) or by the Sertoli cell junctions which form the last of the barriers which separate the seminiferous epithelium from the rest of the body. The function of these barriers is presumably to isolate the seminiferous epithelium and to allow the formation of the unique intratubular environment necessary for the production of fully differentiated spermatozoa. An additional effect of these barriers is to separate the testis into distinct compartments.

Although all three of the barriers described above play a role in this compartmentalization, it is the barrier at the level of the Sertoli cells which has been most widely characterized(Setchell, 1980). It is impermeable to many large molecules, and functionally separates the tubules from the interstitium. The main function of this barrier is not merely the exclusion of certain large molecules, but rather the maintenance of a specific ionic and hormonal environment which enables the complex process of spermatogenesis to be maintained(Waites & Gladwell, 1982). The differences in ionic composition of seminiferous tubule fluid, rete testis fluid and plasma show that the barrier is also capable of maintaining ionic differences within the testis, as the concentration of potassium in seminiferous tubule fluid is more than ten times higher that in plasma, while sodium and chloride concentrations are lower in

seminiferous tubule fluid than in plasma(Setchell, 1980). These observations have been interpreted as providing evidence for the secretion of a potassium and bicarbonate rich fluid into the lumen of the seminiferous tubules. In terms of larger molecules their passage across the Sertoli cell barrier appears to relate more closely to their lipid solubility than to their molecular size(Setchell, 1980) although some specific uptake mechanisms do exist for certain molecules. It is thought that the lipid soluble molecules enter by traversing the Sertoli cells(Setchell, 1980), and that water soluble materials enter via the spaces between the cells, and hence are obstructed by the tight junctions between Sertoli cells(Dym & Fawcett, 1975; Russell, 1978). Specific uptake of some molecules has been demonstrated, but the data is limited to a very few instances. Facilitated diffusion of both glucose and testosterone has been shown(Setchell & Middleton, 1971; Setchell & Main, 1975; Setchell, Laurie, Main & Goats, 1978), and it has been suggested that in situations in which testosterone production is reduced, this mechanism could ensure that any testosterone produced is more likely to enter the seminiferous tubules than the circulation, thus providing a means of conserving testicular levels of testosterone. In this respect it is interesting that when interstitial fluid levels of testosterone are elevated following hCG treatment, then a relatively large proportion of the testosterone produced is secreted into the bloodstream, whilst when testosterone levels are normal, relatively less testosterone is secreted into the bloodstream(Sharpe & Cooper, 1983).

Some molecules enter the Sertoli cells in the form of a permeable precursor which is then transformed into an non-permeable product, as is the case for inositol(Middleton & Setchell, 1972) and

for some amino acids(Setchell, Hinks, Volgmayr & Scott, 1967). The presence of the Sertoli cell barrier has several important implications both with respect to the physiology of the testis itself and in the study of any possible testicular hormones or toxins. The barrier appears during pubertal development(Setchell, Laurie & Fritz, 1980) and this suggests that its functional integrity is closely linked with the production of spermatozoa, and it is therefore interesting to note that an early sign of testicular damage in cryptorchid animals is the appearance of vacuoles in and around the Sertoli cell tight junctions which may lead to the breakdown of the Sertoli cell barrier(Kerr, Rich & de Kretser, 1979b). It has also been suggested(Setchell, 1980) that toxins with specific actions on the seminiferous tubules could be concentrated in a similar manner to the natural metabolite inositol, resulting in a locally toxic effect. However, it should be noted that no such toxins are presently known. When functional, the Sertoli cell barrier protects the haploid germ cells from the immune system, and this may be crucial as surface autoantigens may be expressed for the first time after meiotic division of germ cells(O'Rand & Romrell, 1980). Although it is known that FSH exerts its action on the Sertoli cell, it cannot penetrate the Sertoli cell tight junctions to reach the adluminal portion of the Sertoli cells and it is therefore interesting that FSH receptors appear to be exclusively located on the basal surface membrane of Sertoli cells(Orth & Christensen, 1979).

1.2.1: Organization of Spermatogenesis:

The seminiferous tubules, which are the site of sperm production, are a highly differentiated and organized tissue. Within the tubules, specific cellular associations can be recognized,

and cross sections of fixed seminiferous tubules show several layers of germ cells at different stages of development, combined in a consistent manner, and in a fixed cycle(Perey, Clermont & Leblond, 1961). Thus, spermatids at a given stage of differentiation are always linked with spermatocytes and spermatogonia at a set stage of their respective developmental processes. These stages of development can be classified into a number of different types, and the definition of these types has greatly assisted the study of tubular cell interactions. Originally the spermatogenic wave of the rat was separated into 8 stages(Roosen-Runge & Giesel, 1950), but the most widely accepted classification now separates the wave into 14 distinct stages based on the nuclear morphology of spermatids(Leblond & Clermont, 1952a,b). Further subdivision of the stages shows that a total of 27 sub-stages can be identified(Perey et al, 1961), but due to the ease of identification of the 14 main stages, it is with this system that the majority of so-called stage-dependent investigations have been carried out. In the rat it has been shown that each stage of the spermatogenic cycle generally comes into contact only with the stages numerically adjacent to it(thus stage VIII will always be found between stages VII & IX), and because of this it is possible to dissect out pools of tubules of a specific stage(Parvinen & Ruokonen, 1982). Whilst the type of organization of the spermatogenic wave seen in the rat is found widely amongst other mammals, including the primates(Roosen-Runge, 1977; Chowdhury & Marshall, 1980), man is a notable exception to this rule. In the human testis instead of a sequential wave of spermatogenesis as is seen in the rat, there is a "patchy" organisation of the spermatogenic epithelium, with considerable variation in the stages of adjacent zones of epithelium

(Perey et al, 1961). However, the most recent analysis of the human seminiferous epithelium suggests that, as in the rat, there is a sequential spermatogenic wave which progresses along the length of the seminiferous tubule, but it is organized in a highly complex helical pattern(Schulze & Rehder, 1984).

Based on the classification of the rat seminiferous epithelium described by Leblond and Clermont(1952b), a system of transilluminated stage dissection of whole seminiferous tubules has been developed (Parvinen & Ruokonen, 1982), by which it is possible to distinguish between four broad groups of stages(stages IX-XII, XIII-I, I-VI and VII-VIII), and also to separate out individual stages and, in the case of stage VII, substages as well. Using this system a number of hormonal and enzymatic differences have been described between different stages of the spermatogenic cycle(see Parvinen, 1982 for review, see also section 1.4 below).

1.2.2: Interstitial tissue:

The interstitial tissue constitutes about 16% of the total testis volume, and contains Leydig cells, macrophages, fibroblasts, capillaries and lymphatic vessels or spaces(Christensen, 1975). The Leydig cells form nearly 3% of the total volume of the rat testis, and morphometric analysis has shown that there are approximately 22 million Leydig cells per gram of tissue(Mori & Christensen, 1980). Whilst little is known about the physiological role of testicular macrophages and fibroblasts, the Leydig cell has been more widely studied.

a) Leydig cells:

The major functional component of the interstitial tissue is the Leydig cell, which is the source of the testosterone produced by the

testis. Studies have shown that the major intracellular organelle of the Leydig cell is the smooth endoplasmic reticulum, which is the site of many of the steroidogenic enzymes and comprises 60% of the membrane area of the Leydig cell(Mori & Christensen, 1980). The average Leydig cell has a surface area 3 times that of a sphere of the same volume, suggesting that the surface of the cell is highly microvillous(Mori & Christensen, 1980). By measuring the total testosterone output of the testis in vivo(Free & Tilson, 1973) and dividing by the total Leydig cell number per testis it has been calculated that each Leydig cell secretes an average of 0.44pg of testosterone per day, or just under 11,000 molecules per second(Mori & Christensen, 1980). Since testicular androgens are essential for masculinization and fertility, the role of the Leydig cell in the induction and maintenance of spermatogenesis and fertility is a central one. It is therefore of interest to trace the development and function of the Leydig cell, with particular reference to the parallel development of the seminiferous epithelium since the function of these testicular compartments are closely interlinked and dependent on each other.

i) Leydig cell development: In many mammals there are two distinct generations of Leydig cells with different morphological appearances. The first generation of Leydig cells arises in utero as early as the fifteenth day of gestation in the rat(Picon & Gangnerau, 1980), and disappear at birth or shortly thereafter, and are known as the 'foetal' generation of Leydig cells. The timing of the appearance of the second generation of 'adult' Leydig cells is geared to puberty, so that in the rat it occurs almost simultaneously with the reduction in foetal Leydig cells. However, in man these adult Leydig cells do not appear until between 11-13 years of age(Niemi & Ikonen, 1963;

Niemi & Kormano, 1964; Labrie et al, 1980; Mancini, Vilar, Lavieri, Anderson & Heinrich, 1963). These two generations of Leydig cells have been demonstrated in the rabbit(Gondos, Renston & Goldstein, 1976), hamster(Gondos, Paup, Ross & Gorski, 1974), and pig(van Straaten & Wensing, 1978) and may also be present in other species. Foetal Leydig cells possess LH receptors and respond to gonadotrophins by secreting testosterone(Sharpe, 1982; Huhtaniemi, Korenbrot & Jaffe, 1977).

Adult Leydig cells are thought to differentiate from interstitial mesenchymal cells. As these cells differentiate they undergo a number of changes, including proliferation of organelles involved in the production and processing of secretory compounds, i.e. mitochondria, smooth endoplasmic reticulum and Golgi bodies. The full adult population of Leydig cells is achieved primarily by differentiation from precursor cells, with a lesser proportion being derived directly from dividing differentiated cells(Hooker, 1970; Christensen, 1975). In the rat, as in man, the full adult Leydig cell population is achieved during early to mid-puberty, that is between 32-55 days in the rat(Clegg, 1966; Knorr, Vanha-Perttula & Lipsett, 1981; Pahnke, Leidenberger & Kunzig, 1975; Lording & de Kretser, 1972). This development and differentiation of Leydig cells is controlled by pituitary hormones and can be halted, and to some extent reversed by hypophysectomy(Hooker, 1970). Whilst LH might logically seem to be the hormone most likely to control Leydig cell differentiation, recent studies suggest that FSH is probably more closely involved in the differentiation of Leydig cells than is LH. Thus, it has been shown that high FSH levels during puberty correlate well with the development of Leydig cells(Sharpe, 1982), and using detailed

morphometric evaluation of Leydig cell numbers it has been shown that FSH treatment can increase the number of mature Leydig cells present in prepubertal hypophysectomized rats(Kerr & Sharpe, 1985a,b). Since Leydig cells do not possess FSH receptors, this suggests that FSH dependent Sertoli cell factors may play a major role in the development and maturation of Leydig cells.

ii) Heterogenity of adult Leydig cells: Isopycnic centrifugation of adult rat Leydig cells on Ficoll gradients indicated that there were at least two subpopulations of Leydig cells present in the testis(Janszen, Cooke, van Driel & van der Molen, 1976). Further studies on these Leydig cell subpopulations centred around the possibility that cells might be damaged during isolation causing the production of more than one population. Whilst it is now accepted that some of the cells in the three subpopulations obtained following Percoll density gradient separation represent cells damaged during isolation(Cooke et al, 1983), it is also clear that this observation does not completely explain the presence of multiple Leydig cell populations. Cooke et al(1983) showed that of three Leydig cell populations, each of differing densities, isolated, 50% of the cells in the two less dense populations (I & II) were damaged. All 3 populations specifically bound hCG and produced LH stimulated increases in cAMP production, although levels in band I were low or undetectable. However, LH stimulated testosterone secretion could only be detected in bands II & III. Studies by Payne et al(1980a) showed that Leydig cells isolated on metrizamide gradients form two seperate populations and it has been shown that there is no difference in the number or affinity of Leydig cell LH-receptors between these sub-populations, both having about 50,000 binding sites per Leydig cell

with a Ka of 6.4-6.7 x $10^{9}M^{-1}$(Payne et al, 1980a). Nor do Leydig cells isolated in this manner shift their positions, on the metrizamide gradients used for isolation, following a further period of collagenase digestion(Payne et al, 1980a) suggesting that these sub-populations do not represent damaged Leydig cells. Further studies by the same group suggest that pretreatment in vivo with LH increases testosterone production in vitro by type I Leydig cells, to levels comparable with the responsive type II cells(Payne, Wong & Vega, 1980b); Both cell types suffered loss of LH-receptors during this treatment. However, following collagenase dispersion, of mouse Leydig cells, the number of LH receptors in Leydig cells from the 'less dense' fraction of Leydig cells was decreased(Kerr, Robertson, de Kretser, 1985c). Since this difference was not evident in Leydig cells obtained by mechanical dispersion, it was concluded that such effects were the result of enzymatic dispersion(Kerr et al, 1985c). Whilst this may apply to LH receptor numbers, this does not conclusively disprove the dual population theory, especially in the light of previous data by Payne et al(1980a,b). Furthermore the presence of two morphologically and functionally dissimilar Leydig cell populations in mechanically dispersed cell suspensions(Kerr et al, 1985c) suggests that dissimilarities that are found in the mouse following collagenase dispersion are not wholly attributable to enzymatic damage. Further evidence in support of Leydig cell heterogeneity is provided by studies on in vivo dissimilarities in the morphology of Leydig cells. For example, it has been shown that peritubular Leydig cells are larger that those situated in perivascular and central positions within the interstitium, and furthermore that those cells positioned adjacent to stages VII-VII of the spermatogenic cycle are larger that

those positioned elsewhere(Bergh, 1982; 1983b). Therefore, since the possibility of damage to Leydig cells during isolation has been considered, and since in vitro findings have now been supported by those in vivo, the existence of a heterogeneous Leydig cell population would appear to be established. However, whether the populations seperated in vitro truly represent those seen in vivo, and the physiological significance of such differences seen in vitro is not yet known. Nor is it known why different isolation procedures should result in the isolation of two or three Leydig cell populations(see above).

iii) Hormonal regulation of Leydig cells: LH receptors increase dramatically in numbers from about 21 days postnatally to full maturity in the rat(Sharpe, Hartog, Elwood & Brown, 1973) principally due to an increase in Leydig cell numbers(Sharpe, 1982). This rise is hormone-dependent and is rapidly reversed by both hypophysectomy and LHRH immunization(Frowein & Engel, 1975; Sharpe & Fraser, 1979), although short term deprivation of gonadotrophins in the adult rat has no effect(Hauger, Chen, Kelch & Payne, 1977) suggesting a possible role for other pituitary hormones (e.g. prolactin). Following hypophysectomy the presence of two Leydig cell populations with different LH receptor numbers has been reported, with approximately 50% of Leydig cells retaining normal LH receptor numbers(Hsueh, Dufau, Katz & Catt, 1976; Dufau, Hsueh, Cigorraga, Baukal & Catt, 1978). As with Leydig cell numbers the rise in LH receptor numbers correlates well with the serum levels of both prolactin and FSH during development in the rat, suggesting that these hormones may be involved in Leydig cell development(de Jong & Sharpe, 1977; Negro-Vilar, Krulich & McCann, 1973; see Sharpe, 1982 for

review). Similar rises in FSH during puberty occur in the human(Swerdloff & Heber, 1981).

It is now widely accepted that LH down-regulates its own receptors on the Leydig cell(See Sharpe, 1982 for review). This down-regulation is not due merely to receptor occupancy and is maximal about 48 hours after injection of hormone. The injected dose has little effect on the time course of receptor loss(Sharpe & McNeilly, 1978; Tsuruhara, Dufau, Cigorraga & Catt, 1977), unless pharmacological doses of hCG(500 I.U.)are used(Haour & Saez, 1978). Following receptor loss, levels are gradually restored over a number of days, depending on the dose of hormone(LH or hCG) injected(Haour & Saez, 1977). Although these studies were carried out in the rat there is evidence that pretreatment with hCG causes LH receptor down-regulation in man (Sharpe, Wu & Hargreaves, 1980). The loss of LH receptors has been shown to involve internalization of the hormone-receptor complex by aggregation of the hormone-receptor complexes in coated pits and subsequent microphagocytosis of the vesicles at the cell surface. The vesicles thus formed then fuse with lysosomes and the bound hormone is degraded. It is possible that, prior to fusion with lysosomes, the receptors are dissociated from the hormone and that these receptors are then recycled(Ascoli, 1984). It has been shown that prior to down-regulation of receptors there is an initial increase in LH receptors on the Leydig cell following LH stimulation (Sharpe, 1982; Hsueh, Dufau & Catt, 1977). This may induce initial hyperactivity of Leydig cells in response to LH stimulation, although the function and relevance of this change and of the subsequent down-regulation of LH receptors remains unclear. Moreover, not only has the physiological significance of the down-regulation of LH-

receptors been questioned(Payne et al, 1980b), and it is suggested that desensitization of Leydig cell steroidogenesis does not occur except with a single administration of a high, nonphysiological dose of LH or hCG. Moreover, additional, 'upstream', mechanisms for the desensitization of LH receptors have been proposed(Dix, Habberfield & Cooke, 1984). Working with cells isolated from rat Leydig cell tumours it has been shown that following administration of LH an 'uncoupling' of the LH-receptor interaction with the guanine nucleotide regulatory protein involved in activation of adenylate cyclase occurs, without the loss of LH-receptors from the cell surface(Dix, Schumacher & Cooke, 1982). These changes are dependent on protein synthesis, as is the subsequent decrease in LH-receptors, although, both are preceded by an initial rise in LH-receptor numbers, which is probably not dependent on protein synthesis(Dix & Cooke, 1981). It now appears that treatment of tumour Leydig cells with LH results in a two stage densensitization of the LH-receptor action on these cells. First, there is a lesion between the LH-receptor and the guanine nucleotide regulatory proteinas described by Dix et al(1982), which is independent of cAMP. Secondly via a cAMP mediated desensitization of rat Leydig cell adenylate cyclase which occurs distal to the regulatory protein(Dix et al, 1984). The physiological role of these and other LH-receptor regulatory systems is not yet known.

Finally it is known that prolactin increases LH-receptor numbers in a number of species, although again the physiological significance of this event is unclear(See Sharpe, 1982 and below).

iv) Leydig cell steroidogenesis: The enzymes involved in Leydig cell steroidogenesis(Hall, 1970; Eik-Nes, 1975; Ewing &

Brown, 1977), show a similar developmental pattern to that of Leydig cells and LH receptors. The majority of steroidogenic enzymes reach their peak activity at between 30-35 days postnatally(Inano, Hori & Tamaoki, 1967; Payne, Kelch, Murono & Kerlan, 1977). The major exception to this rule is the final enzyme in the steroidogenic pathway for testosterone, 17β-hydroxysteroid dehydrogenase. This enzyme starts to increase in activity at between 30-40 days of age and this increase coincides with an increased ability of the testis to secrete testosterone(see Sharpe, 1982 for review; Inano et al, 1967; Payne et al, 1977).

The relative inability of the immature rat testis to secrete testosterone has been linked to the high activity of another steroidogenic enzyme, 5α-reductase(Nayfeh, Barefoot & Bagget, 1966; Folman, Sowell & Eik-Nes, 1972), which may be stimulated by the high levels of FSH in serum observed at this time(Folman et al, 1972), resulting in the secretion of the less androgenic steroids, androstanediol and androsterone(Purvis, Clausen & Hansson, 1978; Podesta & Rivarola, 1974). The maturation of the rat involves the development of a greatly increased ability of the Leydig cells to secrete testosterone, although the total androgen output of the testis may actually be decreased(Purvis et al, 1978).

b) Macrophages:

Macrophages are known to be present within the testes of a number of species, including mice(Ohata, 1979) hamsters(Wing & Lin, 1977), dogs(Connell & Christensen, 1975) rats(Christensen & Gillim, 1969) and men(see Kerr & de Kretser, 1981 for review). Their precise function is unclear, although radiolabelling techniques have shown in the rat that they remain within the testis and do not rapidly turn over

(Miller, Bowman & Rowland, 1983). Testicular macrophages are closely associated with Leydig cells in both the rat(Miller et al, 1983; Christensen & Gillem, 1969)and dog(Connel & Christensen, 1975). Miller et al(1983) have shown that processes extending from the Leydig cells are closely associated with 'coated' pits within the macrophage surface membranes, and these pits may be associated with the transport of waste materials, steroids or other Leydig cell metabolites. Macrophages from hypophysectomized animals have been shown to accumulate FSH, although in untreated animals they do not show this accumulation(Orth & Christensen, 1977).

c) Fibroblasts:

The function of testicular fibroblasts is not known, although the deposition of connective tissue is presumably mediated by these cells. Recently, human fibroblasts have been shown to possess the ability both to mature into Leydig cells following hCG stimulation, and also during this maturation, the precursors have been shown to produce quantitatively significant amounts of testosterone(Chemes, Gottleib, Pasqualini, Domenichini, Rivarola & Bergada, 1985). Whether this identifies the fibroblast as the only possible Leydig cell precursor within the human testis, and whether these results are applicable to other species, remains to be seen.

1.3: Pituitary hormone control of the testis:

Once pituitary extracts could be separated into LH and FSH, studies upon the relative roles of these hormones and of other pituitary and peripheral hormones within the reproductive tissues became a priority in the understanding of testicular function and control(See above). The properties of these hormones are now widely studied as are their relative mechanisms of action.

1.3.1: Luteinizing hormone:

Luteinizing hormone, synthesized in the pituitary gland, was first purified in the 1930's and its availability provided the means of demonstrating that LH acted upon the Leydig cells(Greep, Fevold, & Hisaw, 1936), as it was shown that this preparation could maintain normal Leydig cell morphology after hypophysectomy. It was not until the 1960's that it could be shown conclusively that LH action was restricted to the interstitial tissue of the rat(Mancini, Castro & Seigur, 1967). During the same period studies demonstrating *in vivo* localization of iodinated LH and the *in vitro* binding of LH by rat testis homogenates were also carried out(de Kretser, Catt, Burger & Smith, 1969; de Kretser, Catt & Paulsen, 1971). The majority of early studies confirmed that LH had no direct effects on the seminiferous tubule, or showed effects in circumstances likely to be associated with Leydig cell contamination of the seminiferous tubule preparations(Cooke, Rommerts, van der Kemp & van der Molen, 1974). LH has been shown to bind to a cell surface receptor on the Leydig cells (Hsueh, Dufau, Katz & Catt, 1976) although some earlier studies had suggested that LH was present on intracellular hormone receptor complexes(Castro *et al*, 1970; de Krestser *et al*, 1971). It is now assumed that there is a rapid turnover of LH/hCG receptors, and that, following binding, the receptor-hormone complex is internalized and the receptor either recycled or degraded within the cell(Ascoli, 1984). The specificity of the LH/hCG receptors has been widely characterized(Catt, Dufau & Tsuruhara, 1971; 1972a; Leidenberger & Reichert, 1972; Catt & Dufau, 1973) and its affinity for hCG in the male rat is reported as 3×10^{-10}M(Catt *et al* 1972; Leidenberger & Reichert, 1972) which falls within the range necessary for it to be

able to detect circulating levels of LH(10^{-9}-10^{-10} M in male rats).

Available evidence suggests that LH activates adenylate cyclase, (Cooke, Lindh & Janszen, 1977a,b; Janszen, Cooke & van der Molen, 1977) leading to the production of cAMP which then stimulates protein kinase(Cooke, Lindh & Janszen, 1976; Cooke & van der Kemp, 1976) and protein synthesis and/or phosphorylation(Cooke et al 1975; Janszen, Cooke, van Driel & van der Molen, 1976; Mendelson, Dufau & Catt, 1975). However it is not certain that cAMP has an obligatory role since many investigations show there is a wide difference in the amounts of LH required to produce stimulation of steroidogenesis and cAMP production respectively(van der Molen et al, 1979). Thus, it has been shown in studies using LH and cholera toxin that at low levels of LH stimulation there is no increase in cyclic AMP levels within the Leydig cell, while steroidogenesis can increase to half the maximum rate before any increase in cyclic AMP can be detected. This may indicate that cyclic AMP is involved in the action of LH but that other factors may be present during stimulation with low levels of LH or hCG(Cooke et al, 1977b). However, it can be shown that over a range of LH doses, protein kinase activation and testosterone production are closely linked, the only discrepency being in the dose of LH required to produce maximal stimulation of either steroidogenesis or protein kinase activity, because when steroidogenesis is stimulated maximally further stimulation of protein kinase by LH is possible(van der Molen et al, 1979). The apparent discrepancy between these results can be explained by the observations of Dufau, Tsuruhara, Horner, Podesta & Catt(1977b), who showed a close correlation in hCG stimulated Leydig cells between testosterone production and the number of occupied cAMP sites upon the regulatory subunit of the Leydig cell protein kinase.

These results suggest that although there is no detectable free cAMP present in these cells, nonetheless cAMP is involved in this process and the reason for the failure to detect its presence is that it is closely bound to the regulatory subunit of protein kinase.

The involvement of protein synthesis has been shown in studies in which the response of Leydig cells to LH has been blocked by cycloheximide(Cooke et al, 1975). Following stimulation of Leydig cells with LH, cycloheximide was added and testosterone production declined to control levels, this decline following first order kinetics, with a half-time of 13 minutes. This suggests that testosterone production is dependent on continuous synthesis of a protein with a short half-life (van der Molen et al, 1979). Inhibitors of RNA synthesis can also cause inhibition of testosterone production following LH stimulation, suggesting that de novo synthesis of mRNA coding for this or other proteins may also be involved in the action of LH on testosterone production(van der Molen et al, 1979). On stimulation of the Leydig cell with LH two proteins can be detected which are newly synthesized as a result of this stimulation(Janszen et al, 1977). The first has a molecular weight of 21 kDaltons, a half life in excess of 30 minutes and is not sensitive to cycloheximide. The second has a molecular weight of 33 kDaltons and a half life of about 11 minutes and is sensitive to cycloheximide. It has been suggested that there is a larger precursor for the 33 kdalton protein and that a pool of this precursor exists from which the smaller is rapidly produced upon stimulation of the Leydig cell by LH(Janszen et al, 1977). LH has also been shown to rapidly stimulate the phosphorylation of a number of Leydig cell proteins(Cooke et al, 1977b), as within 5 minutes of stimulation by LH three proteins of molecular weights 14.3, 57 & 77.6

kDaltons were phosphorylated. This is consistent with data showing that over the same time period protein kinase activity is increased (Cooke & van der Kemp 1976, Cooke et al 1976). Whilst dibutyryl cAMP mimics this action of LH on the Leydig cell, no effect of FSH or testosterone was demonstrated in this system(Cooke et al, 1977b). The endpoint of the action of LH appears at present to be the regulation of mitochondrial cholesterol side chain cleavage involving the cytochrome P450 system.(van der Vusse, Kalkman & van der Molen, 1975).

1.3.2: Follicle Stimulating Hormone:

Both FSH & LH are known to be required for the successful initiation of spermatogenesis in immature animals or those in which the testes have regressed following hypophysectomy(Steinberger, 1971; Lostroh, 1963; Go, Vernon & Fritz, 1971). However the actions of these hormones are rigidly separated within the testis, FSH having direct effects only on the seminiferous tubules, whilst LH acts only on the Leydig cells(Dorrington, Roller & Fritz, 1974). FSH binding was first characterized using tritiated hormone preparations(Means & Vaitukaitis, 1972; Schwartz, Bell, Rechnitz & Rabinowitz, 1973; Miyachi & Inomata, 1974; Bhalla & Reichert, 1974), and binding to testes from rats of all ages could be demonstrated although significantly more FSH was bound in immature animals. This binding was shown to be primarily associated with the plasma membrane(Means, 1973; Davies, 1981) and could only be demonstrated with membranes prepared from the seminiferous tubules, with no binding to interstitial cells(Means & Huckins, 1974). The FSH receptor has been solubilized in detergent and isolated; a small water soluble component, of either newly synthesized or internalized receptors has

also been identified(Dufau, Ryan & Catt, 1977a). The binding of FSH to plasma membranes has been shown to correlate closely with the activation of a membrane bound adenylate cyclase system(Means, 1973; Davies, 1981) and this has been shown to be specific for FSH(Braun & Sepsenwol, 1974). Activation of adenylate cyclase then rapidly activates protein kinase(Means, 1973; Davies, 1981; Tindall & Means, 1976). Bound FSH does not appear to be degraded on the receptors as bioactive FSH has been recovered from receptor preparations(Means & Huckins, 1974). The stimulation of protein kinase by FSH is age-dependent, and there is a marked decline in FSH stimulated kinase activity after 16 days of age, such that by 30 days of age no effect of FSH can be demonstrated(Means & Huckins, 1974).

FSH binding has also been shown to be age dependent with the greatest capacity for binding occurring between 10 and 16 days of age in the rat(Salhanick & Wiebe, 1980), although the number of receptor sites per testis is reported to be greatest in mature animals(Thanki & Steinberger, 1978). FSH binding also changes locally within the testis according to the stage of the spermatogenic cycle of the associated germ cells(Parvinen, Marana, Robertson, Hansson & Ritzen, 1980). However, hypophysectomy restores the sensitivity of the testis to FSH(see above). This decrease in activity and its return can be associated with the appearance of a specific phosphodiesterase isozyme(Monn, Desautel & Christiansen, 1972; Christiansen & Desautel, 1973). As with LH the levels of FSH required to increase protein kinase activity significantly are much lower than those required to raise cAMP levels(Means & Huckins, 1974).

To further localize within the seminiferous tubules the site of FSH binding, studies were performed using animals in which the testes

had been irradiated to form Sertoli-cell-only tubules, and using this technique, it has been shown that the level of binding of FSH to the tubules is almost identical to that seen in normal animals, proving that the Sertoli cell is the site of action of FSH(Means & Huckins, 1974). The Kd for FSH binding has been shown to be about 10^{-10}M(Means & Huckins, 1974; Dorrington et al, 1974).

FSH has a wide range of morphological effects on the immature testis, including increasing the number of Sertoli cells(Courot, 1965; Davies & Lawrence, 1978), which can also explain the increase in size of the remaining testis following early hemicastration (Cunningham, Tindall, Huckins & Means, 1978). FSH increases the number of spermatogonia in immature testes by reducing the number of degenerating spermatogonia(Davies & Lawrence, 1978; Means, 1975), an effect which may be explained by the FSH-induced increase in DNA synthesis seen in germ cells of hypophysectomized immature rats (Ortavant, Courot & Hochereau-de Riviers, 1972). Physiologically, the period of development when FSH levels are highest correlates with the period of marked increase in the number of Sertoli cells and spermatogonia up to 40 days of age in the rat(Mackinnon, Puig-Duran & Laynes, 1978). FSH may also synergize with endogenous androgens to induce the formation of primary spermatocytes and of spermatids during maturation (Clausen, Purvis & Hansson, 1979). Yet despite this wide range of actions in the immature animal, experiments in intact adult rats support the theory that FSH is not absolutely essential for the maintenance of spermatogenesis once it is successfully established (Davies, 1981).

One of the major known effects of FSH on the testis is to stimulate production of an androgen binding protein(ABP) by the

Sertoli cell(Hansson, Ritzen, French & Nayfeh, 1975). Both FSH and testosterone have been shown to stimulate the synthesis of ABP independently(Fritz, 1978), but there are also reports that this "FSH" effect is a result of elevated testosterone concentrations within the testis(Tindall & Means, 1976), and that the primary regulator of ABP synthesis within the testis is testosterone. This latter theory is supported by results showing that only treatments which elevate testicular testosterone levels also increase ABP levels, and that purified FSH has no effect on ABP, whilst LH treatment causes increased levels(Tindall & Means, 1976). Linked to this are reports that pretreatment of immature rats with hCG increases oestradiol secretion following a subsequent stimulus of FSH, by increasing the intratesticular concentration of substrate(testosterone) for the synthesis of oestradiol within the seminiferous tubules(Pomerantz, 1984).

FSH secretion by the pituitary is partially controlled by a testicular hormone inhibin. The existence of inhibin is now widely accepted, although for may years there were doubts as to the relevence of this hormone. The existence of inhibin was postulated as early as 1923, and the compound named in 1932(See Baker, Burger, de Kretser, Findlay, Hudson, Lee & Tsonis, 1983; Steinberger, 1983; Main, Davies & Setchell, 1979 for historical reviews). Early experiments were unsucessful in seeking to elucidate the structure, function and physiological role of inhibin, and this led to speculation about its existence. However, more recent work has shown that inhibin not only exists but that it is probably the major feedback regulator of pituitary FSH secretion(Baker et al, 1983; Steinberger, 1983; Main, Davies & Setchell, 1979).

Inhibin is a water soluble protein(Means & Huckins, 1974; Dorrington, Roller & Fritz, 1974), but despite a decade of effort by different workers, its exact structure has yet to be described. Inhibin is heat labile(Steinberger, 1983) and probably has a molecular weight between 30-60 Kdaltons although values from 2-2,000 Kdaltons have been reported(Baker et al, 1983 for review). A possible explanation for this heterogeneity is the suggested ability of inhibin to form complexes with other proteins or to polymerize with other inhibin molecules(Baker et al, 1983).

The major role of inhibin is accepted as the feedback regulation of FSH secretion, both in male and female animals and man (Baker et al, 1983; Steinberger, 1983; Main, Davies & Setchell, 1979). In males the site of production is the Sertoli cells, and there are many reports of inhibin production by both seminiferous tubules and isolated Sertoli cells(Eddie, Baker, Dulmanis, Higginson & Hudson, 1978; Steinberger, 1979; de Jong, Welschen, Hermans, Smith & van der Molen, 1979; Steinberger & Steinberger, 1976; see also Baker et al, 1983; Steinberger, 1983; Main, Davies & Setchell, 1983 for reviews). Inhibin has also been shown to be present in follicular fluid, and is secreted from granulosa cells(de Jong & Sharpe, 1976; Baker et al; 1983, Steinberger, 1983; Main, Davies & Setchell, 1983). Inhibin-like activity has also been demonstrated from other sources, including human seminal fluid(Franchimont, Demoulin, Verstraelen-Proyard, Hazee-Hagelstein & Tunbridge, 1979), bull spermatozoa(Lugaro, Casellato, Manera, Pasta, Bacigalupo & Lauria, 1979), rete testis fluid(Steinberger & Steinberger, 1976; Baker et al, 1983) and serum(Baker et al, 1983). However, the physiological significance of the seminal plasma inhibin must now be open to doubt

in view of a recent demonstration that it is prostatic in origin (Beksac, Khan, Eliasson, Skakkebaek, Sheth & Diczfalusy, 1984).

Inhibin has been shown to act both at the hypothalamic level, modulating the action of LHRH(Labrie et al, 1978) but also directly on the pituitary gland to modify the synthesis and secretion of FSH (Baker et al, 1983). Investigations aimed at characterizing the receptors for inhibin have not yet succeeded due largely to the lack of pure inhibin preparations(Baker et al, 1983).

1.3.3. Prolactin:

Prolactin binding in the rat testis has been demonstrated(Aragona & Friesen, 1975; Bartke, Smith, Michael, Peron & Dalterio, 1977), and these binding sites are known to be localized to the Leydig cell (Aragona, Bohnet & Friesen, 1977; Charreau, Attramadal, Torjesen, Purvis, Calandra & Hansson, 1977). During development in the rat prolactin receptors increase about 3 fold between days 20 and 45(see Sharpe, 1982), this change being more or less co-incident with reported changes in serum prolactin over this period(Dohler & Wuttke 1974; 1975). Both LH and prolactin receptors are reduced in prolactin deficient dwarf mice(Bohnet & Friesen, 1976) and also following treatment of normal rats with hCG(Davies, Katikineni, Chan, Harwood, Dufau & Catt, 1980). Treatment of rats with prolactin causes an increase in Leydig cell LH-receptor numbers at the same time as causing a marked decrease in the number of prolactin receptors (Morris & Saxena, 1980). Although numerous reports of LH receptor regulation by prolactin are present in the literature(see Sharpe, 1982 for review) the role of prolactin during maturation is still not clear. Implantation of pituitaries, to cause hyperprolactinaemia in immature male rats, increased serum testosterone and the development

of male accessory glands(de Jong & van der Schoot, 1979) and these results were confirmed by studies using injected prolactin(Baranao et al, 1981). Suppression of prolactin has been shown to inhibit the maturation of spermatocytes into spermatids in immature rats(Nag, Sanyal, Ghosh, & Biswas, 1981), but no effect on spermatogenesis is seen in the mature state(Alger, Pfeiffer & Bocabella, 1975). Thus prolactin has now been established as playing a role in the development of the testis in the immature animal, but its role in the adult is yet to be fully explained.

1.3.4: Effects of peripheral hormones other than the gonadotrophins:

i) Growth hormone:

Growth hormone has been shown to partially maintain LH receptor levels following hypophysectomy in both adult and immature rats(Bambino, Schreiber & Hsueh, 1980; Zipf, Payne & Kelch, 1978), and to increase LH receptor numbers in seasonally regressed hamsters(Bex, & Bartke, 1978). This effect is mediated independently of a similar prolactin effect(See Sharpe, 1982 for review). Growth hormone also increases testicular responsiveness to LH in hypophysectomized rats (Odell & Swerdloff, 1976). Despite this evidence for the action of growth hormone in both adult and immature animals its role in the regulation of Leydig cells under physiological conditions is not clear.

ii) Insulin:

Although many studies have shown that insulin deprivation results in impaired Leydig cell function and reduced testosterone secretion, until recently no evidence for a direct effect of insulin on Leydig cells could be found(Sharpe, 1982). Studies using rat testicular cells have shown that insulin may play an important role

in the direct augmentation of testicular androgen production, as it was shown that treatment of cells with insulin increased their hCG stimulated testosterone production without altering DNA or protein content or increasing cell numbers(Adashi, Fabics & Hsueh, 1982). Many of the changes noted in diabetic rats and men may be due either to depletion of peripheral insulin and/or gonadotrophin supply, as both LH levels and the pituitary response to LHRH are reduced(Cusan, Belanger, Seguin & Labrie, 1980; see also Sharpe 1982 for review), or to local factors released as a result of seminiferous tubule damage(See for example, Rosenmann, Palti, Teitelbaum & Cohen, 1974; or Sharpe, 1982 for review).

iii) Thyroid Stimulating Hormone(TSH):

High affinity receptors for thyroid stimulating hormone have been reported in the rat testis(Amir, Sullivan & Ingbar, 1978) but there are no reported effects of TSH on the Leydig cell.

iv) Melatonin and Serotonin:

Both these hormones have been shown to have direct inhibitory effects on Leydig cell steroidogenesis in vitro by inhibiting the activities of Leydig cell 17α-hydroxylase and 17-20 desmolase(Ellis,1972). These results are supported by further *in vitro* effects of melatonin on testicular steroidogenesis(Kalla, 1979) and also of serotonin *in vivo*(Kalla, 1979). Some effects were also seen *in vivo* but these studies are hampered by the possibility of vasoactive effects of these hormones(See Sharpe, 1982).

1.4: Local control of the testis:

The testis is a highly complex tissue, containing numerous different cell types. The seminiferous tubule is surrounded by a basal lamina comprised of two acellular layers, and also by a

layer of peritubular cells, which have been shown to be contractile in nature(rat; Clermont, 1958; human; Ross, 1966). This myoid cell layer has also, more recently, been shown to contain numbers of cells which are not contractile comprising of mononuclear leukocytes (Mori & Christensen, 1980) and also mesenchymal peritubular cells (Mather, Gunsalus, Musto, Cheng, Parvinen, Wright, Perez-Infante, Margioris, Liotta, Becker, Krieger & Bardin, 1983). However the peritubular myoid layer remains the major cell component within the basal lamina of the seminiferous tubule, and these cells contract in response to oxytocin(see below section 1.4.4) and therefore are presumed to play an active role in the expulsion of tubular contents into the rete testis(Clermont, 1958; Ross, 1966). Interactions between these cell types or between those within the seminiferous tubules or interstitium have not been extensively studied, although they are rapidly becoming a focal point of interest.

1.4.1: Sertoli cell-germ cell interactions:

While Sertoli cell function is obviously affected by other testicular cell types, there should be no doubt that the most profound and important interactions are between the developing germ cells and the Sertoli cells. Firstly, because multiplication, meiosis and translocation of the differentiating germ cells have to occur at precise unvarying time intervals(Leblond & Clermont, 1952a; Parvinen, 1982). Secondly, because all of the basic metabolic requirements of the germ cells have to be supplied by the Sertoli cells, i.e. transferrin, lactate, etc(see below). Thirdly, metabolic requirements of germ cells probably change according to the stage of their development(for example see plasminogen activator below).

i) Lactate production by Sertoli cells:

In studies comparing the effects of glucose and lactate upon the viability and function of spermatocytes and spermatids it has been shown that these cells cannot metabolise glucose and depend upon a supply of lactate for their energy requirements(Jutte, Grootegoed, Rommerts & van der Molen, 1981). Although the X-chromosome linked phosphoglycerate kinase and glucose-6-phospate dehydrogenase enzyme genes are inactivated during meiotic prophase(Monesi, 1965) the presence of autosomally coded iso-enzymes could make up for this deficency and allow normal glycolysis to continue(Vandeberg, Cooper & Close, 1973; Brock, 1977). However, the failure of glycolysis in these cells would be an attractive explanation for their failure to utilise glucose. Further studies have shown that Sertoli cells secrete lactate into culture medium and that this lactate can be utilised by spermatocyctes in culture (Jutte, Jansen, Grootegoed, Rommerts, & van der Molen, 1983; Jutte, Jansen, Grootegoed, Rommerts, Clausen & van der Molen, 1982) by means of a testis-specific isozyme of lactate dehydrogenase(LHD-X) which has an equilibrium biased towards the production of pyruvate and preferentially catalyses the oxidation of lactate to pyruvate (Meistrich, Trostle, Frapart & Erickson, 1977; Blanco, Burgos, Gerez de Burgos & Montamat, 1976). It would appear therefore, that Sertoli cells supply spermatocytes and spermatids with energy in the form of lactate which is subsequently oxidised to pyruvate for use in the tricarboxylic acid cycle to produce ATP.

ii) Sertoli cell regulators of mitosis and meiosis:

Cell divisions within the seminiferous tubule must be closely controlled by the Sertoli cell as they have to occur at specific

stages of the spermatogenic cycle. It has been shown that seminiferous tubules secrete a mitogenic factor in the mouse, rat, guinea pig and calf(see review by Bellve & Feig, 1984) which may regulate the mitotic divisions of the stem-cell type A spermatogonia, through to the formation of primary preleptotene spermatocytes. It has been shown that the Sertoli cell is the principal source of the seminiferous growth factor(SGF), and it has been suggested that this factor may act synergistically with transferrin to promote spermatogonial replication(Bellve & Feig, 1984). SGF has been purified and characterized, and is a polypeptide of about 15,700 daltons molecular weight, which is hydrophilic and anionic(Bellve & Feig, 1984).

The characterization of a meiosis regulator in females(Tsafriri, Dekel & Bar-Ami, 1982) led to investigations in the male for similar substances. Early studies suggested that both meiosis-inducing and meiosis-preventing substances might interact during puberty to allow germ cell divisions and the initiation of complete spermatogenesis (Grinsted, Byskov & Andreasen, 1979; O & Baker, 1976). More recently the presence of both meiosis-inducing(MIS) and meiosis-preventing substances(MPS) has also been reported within the male reproductive organs of man(Grinsted & Byskov, 1981). Previously MIS and MPS had only been identified from foetal tissues(Grinsted, Byskov & Andreasen, 1979; Grinsted & Byskov, 1981; O & Baker, 1976), but in man MIS was found in adult as well as foetal and pubertal tissues(Grinsted & Byskov, 1981). The most exciting recent data regarding these factors has shown differential secretion of both MIS and MPS during the spermatogenic cycle of the adult rat(Parvinen, 1982), with MIS secretion being maximal during the stages coinciding

with DNA replication of preleptotene spermatocytes prior to meiosis, i.e. stages VII to VIII.

It would appear therefore that both the maturation of oocytes, and the meiotic events of the spermatogenic cycle in the rat are under close control by regulators of both mitosis and meiosis.

iii) Plasminogen activator:

Linked to the control of meiosis(see above) is the transfer of preleptotene primary spermatocytes from the basal compartment of the seminiferous tubules, i.e. posterior to the Sertoli cell tight junctions, to the adluminal compartment above the tight junctions (Russell, 1980). When meiosis begins a new tight junction is formed behind the preleptotene spermatocytes, so that for a short period these cells are isolated from both the basal and adluminal compartments(Russell, 1980). Next the tight junctions anterior to the cell are dissolved and the germ cells are thus incorporated into the adluminal compartment, and this ensures that the haploid cells are isolated in a specialized controlled environment which appears to be essential for their further development(Parvinen, 1982). This transfer commences at the same stage(VIII) as spermiation, and also coincides with a peak in the Sertoli cell secretion of a protease, plasminogen activator(Lacroix, Parvinen & Fritz, 1981). It is suggested that this protease, which is known to be involved in cell migration and tissue restructuring(Lacroix, Parvinen & Fritz, 1981), plays a role in spermiation and/or the migration of preleptotene spermatocytes within the tubules. This suggestion is further supported by results suggesting that in the absence of specific germ cells, the stage VII-VIII secretion of plasminogen activator is abolished(Vihko, Suominen & Parvinen, 1984). The role of testosterone and other hormones in the

control of these processes is unknown.

iv) Other Sertoli cell proteins:

A number of other proteins related to cell growth and maintenance in culture have been reported as Sertoli cell products. It now appears that as a consequence of the blood testis barrier, which originates at the Sertoli cell tight junctions(see above) these cells must provide a number of hormones which are normally provided via the circulation to maintain the germ cell population.

Sertoli cells have been shown to secrete a somatomedin-like peptide(Johnsonbaugh, Ritzen, Hall, Parvinen & Wright, 1982), the levels of which may vary according to the stage of the spermatogenic cycle, with maximal secretion at stages VII and VIII. Somatomedin is also produced by granulosa cells and can be shown to mediate some effects of FSH(Adashi, Resnick, Svoboda & Van Wyk, 1984). Levels of somatomedin production by granulosa cells may be hormonally controlled (Hammond, Barano, Skaleris, Rechler & Knight, 1984).

Ceruloplasmin, transferrin and insulin have also been isolated from Sertoli cell cultures(Skinner & Griswold, 1982; 1983; Perez-Infante, Parvinen, Lahdetie, Wright, Bardin & Mather, 1981; Wright, Musto, Mather & Bardin, 1981). There is evidence that levels of transferrin secreted in vitro may be controlled by FSH, insulin and other hormones(Skinner & Griswold, 1982). Transferrin secretion also varies with age(Perez-Infante et al, 1981).

v) FSH effects:

During the development of the germ cells, responsiveness of Sertoli cells to FSH falls despite increased numbers of receptors (Parvinen, 1982). As has already been described, this fall is due to increased phosphodiesterase activity in the mature Sertoli cell(see

section 1.3.2). Although the adult Sertoli cell responds very poorly to FSH there is a marked cyclicity in the binding capacity of seminiferous tubules for FSH, such that FSH binding is lowest at stages VII-VIII and highest at stage I of the spermatogenic cycle (Parvinen, 1982). The ability of FSH to stimulate cAMP production in the presence of a phosphodiesterase inhibitor also varies in a cyclical fashion, being lowest at stages VII-VIII and rising to a peak at stages I-V(Parvinen, 1982; Parvinen, Marana, Robertson, Hansson & Ritzen, 1980; Gordeladze, Parvinen, Gautvik & Hansson, 1982). This pattern of FSH induced cAMP production closely parallels the cycle of spermatid manganese-dependent adenylate cyclase activity (Gordeladze et al, 1982) which is specific for haploid germ cells. Thus there may be some transmission of the FSH stimulation of the Sertoli cell to the adenylate cyclase of the spermatids. Another haploid cell specific enzyme, protein carboxyl methylase has a cyclical distribution almost identical to that of manganese-dependent adenylate cyclase(Parvinen, 1982; Cusan, Gordeladze, Parvinen, Clausen & Hansson, 1981), and both these enzymes are thought to have a role in regulating subsequent sperm motility(Gagnon, Axelrod, Musto, Dym & Bardin, 1979; Bouchard, Gagnon, Phillips & Bardin, 1980). Closely correlated with these enzyme activities is the formation of spermatid bundles in stages XII-I and their subsquent migration to become closely associated with Sertoli cell nuclei in bundles at stages II-V (Russell, 1980). These migratory processes may be regulated by cytoskeletal components of the Sertoli cell(Parvinen, 1982), which may be regulated by cyclic AMP levels, which are controlled in turn by FSH (Means, Dedman, Tindall & Welsh, 1978). It is therefore of interest that the rise in adenylate cyclase and protein carboxyl-methylase

activity correlates well with the increased FSH sensitivity, and that at stage VI there is a rapid fall in adenylate cyclase activity linked with a rise in activation of a cAMP phosphodiesterase(Parvinen, 1982; Gravis, 1978). It is thought that these activities may be linked to normal spermatid development and spermiation, since spermiation is inhibited by high cAMP levels(Gravis, 1978).

1.4.2: Sertoli cell-Peritubular cell interactions:

It has recently been shown that peritubular cells interact in a number of ways with Sertoli cells. When cultured, isolated Sertoli cell aggregates grow in monolayers, but when such cells are plated onto a monolayer culture of peritubular myoid cells they form tubular or ball-like structures(Cameron & Markwald, 1981; Bols, Bowen & Byrne, 1984). This effect is specific for peritubular-Sertoli cell cultures, although some reports suggest that other cell types can partially maintain Sertoli cell function or induce similar morphological effects, but that only peritubular cells perform both functions simultaneously(Tung & Fritz, 1980). Peritubular cells have also been shown to cooperate metabolically with Sertoli cells in culture, producing an increase in Sertoli cell ABP production, in both FSH-stimulated and untreated Sertoli cells(Hutson & Stocco, 1981). Further studies on the form of metabolic cooperation between Sertoli cells and peritubular cells have shown that the latter secrete a wide range of proteins, although studies on these proteins are hampered by the inability to obtain pure peritubular cell cultures(Mather, Gunsalus, Musto, Cheng, Parvinen, Wright, Perez-Infante, Margioris, Liotta, Becker, Kreiger & Bardin, 1983). Peritubular cells can transfer RNA or RNA precursors to Sertoli cells in vitro only when the two cell types are actually in contact, and the same studies have shown that the

increase in ABP secretion is not maintained unless the two cell types are in contact. Whether the transfer of RNA is related to ABP production remains to be proven(Hutson, 1983).

The presence of peritubular cells in co-culture with Sertoli cells enhances ABP production by Sertoli cells following stimulation with testosterone. It has been further demonstrated that the peritubular cells secrete a compound into culture medium(as shown by incubating Sertoli cells in peritubular cell conditioned medium) which stimulates Sertoli cell ABP production and furthermore that this compound is modulated by testosterone(Skinner & Fritz, 1985).

1.4.3: Peritubular leukocyte changes:

Cyclic variations in the number of mononuclear leukocytes within the peritubular membrane and also in the number of mitotic figures in this cell population have been described(Mori & Christensen, 1980). There is also a cyclic variation in the frequency of degeneration of these cells, with the number of mononuclear leukocytes peaking at stages XIV-I and IX-XII of the spermatogenic cycle, while the number of mitotic figures peaks at stages VI and XIV. The data suggests that these cells turnover rapidly with a "cycle" of similar length to that of the spermatogenic wave(i.e. 12 days vs 13.3 days, Mori & Christensen, 1980). The functional significance of these cells is still unresolved, but as their cyclicity is so closely linked with that of the seminiferous epithelium then it seems likely that some form of communication takes place between these compartments.

1.4.4: Leydig cell-Peritubular cell interactions:

It has been shown that oxytocin is present in testicular extracts from rats, bulls and men and that following mating

behaviour in the ram, bull and pony, oxytocin is released(see Wathes, 1984 for review). This release of oxytocin could produce contractions of the seminiferous tubules and expulsion of tubular contents from the testes, since oxytocin has been shown to produce contractions of seminiferous tubules(Wathes, 1984 for review). The tunica propria and albuginea of the testis are also said to increase their contractile activity following oxytocin treatment, as are the epididymis & vas deferens in the rabbit(see Wathes, 1984 for review). These effects are also linked with increased sperm and semen output following oxytocin treatment, suggesting that the role of this hormone is to facilitate the expulsion of spermatozoa from the testis into the epididymus(Wathes, 1984 for review), presumably by stimulating contraction of the myoid layer surrounding the seminiferous tubules.

1.4.5: Sertoli-Leydig cell interactions:

A) Morphological evidence:

Most of the available evidence for Sertoli-Leydig cell interactions is indirect and is derived from two major experimental approaches: First, the effect that damage to the seminiferous epithelium has on the Leydig cell, and second the effects of FSH on Leydig cell structure or function(Sharpe & Rommerts, 1983).

Following the induction of damage to the seminiferous epithelium by a range of techniques, and subsequent impairment of Sertoli cell function and of fertility, it has been shown that marked alterations in Leydig cell morphology occur, including hyperplasia, hypertrophy of cellular organelles and enhanced steroidogenic capacity coupled with decreased LH receptor numbers(Sharpe & Rommerts, 1983; Sharpe, 1982).

i) Cryptorchidism: Cryptorchidism involves either the failure of the

testis to descend from its intra-abdominal position during development or the experimental relocation of the testis into the abdomen during surgery. Following surgical induction of cryptorchidism in adult rats it has been shown that there is rapid degeneration of germ cells and significant changes in the structure and function of Sertoli cells (Kerr et al, 1979b). These changes occur within 5-7 days and include the accumulation of lipid droplets and vacuoles within the Sertoli cells and modifications of the Sertoli cell tight junctions with vacuoles appearing within the region of these junctions. This disruption of the Sertoli cell junctions may be indicative of an alteration in the permeability of the blood-testis barrier and in keeping with this there is evidence suggesting that such changes do occur(Hagenas, Ploen, Ritzen & Ekwall, 1977). During longer periods of cryptorchidism there is collapse of the seminiferous tubules due to depletion of germ cells(Kerr et al, 1979b). There is also a decrease in ABP production during the first four weeks of cryptorchidism(Kerr et al, 1979b). In bilaterally cryptorchid rats, these changes include increases in serum LH and FSH levels(Risbridger, Kerr, Peake, Rich & de Kretser, 1981b). Further studies have shown that changes in Sertoli cell FSH receptors, ABP production and testicular fluid production occur within two days following induction of cryptorchidism and that a progressive impairment of these functions occurs thereafter(Jegou, Risbridger & de Kretser, 1983b). Testicular inhibin content is also markedly reduced within seven days of cryptorchidism and falls to about 10% of normal levels after six weeks(Au, Robertson & de Kretser, 1983).

Following this evidence of impaired Sertoli cell function in cryptorchid rats evidence has shown that the Leydig cells are also

affected. Leydig cells in the abdominal testis of unilaterally cryptorchid animals have been shown to be significantly increased in size in the presence of normal serum LH levels(Risbridger, Kerr & de Kretser, 1981c), and ultrastructural changes in the Leydig cells during this treatment include increases in smooth and rough endoplasmic reticulum, mitochondria and Golgi apparatus, all changes indicative of increased Leydig cell steroidogenic capability(Risbridger, Kerr & de Kretser, 1981c). Some mitotic figures in Leydig cells were also seen, suggesting that there may be an increase in Leydig cell numbers (Risbridger, Kerr & de Kretser, 1981c). Isolated Leydig cells from cryptorchid animals have been shown to be hyper-responsive to hCG stimulation *in vitro*(Sharpe, Cooper & Doogan, 1984; Risbridger, Kerr & de Kretser, 1981c), yet *in vivo* reduced levels of testosterone have been found in both testicular interstitial fluid and spermatic venous blood(Sharpe *et al*, 1984). This suggests that despite their increased responsiveness *in vitro*, the response of the Leydig cells *in vivo* is impaired in some way by cryptorchidism. Since no comparable changes occur in the scrotal testes of unilaterally cryptorchid rats, these effects must be due to some local control mechanism. It is interesting, therefore that in addition to the changes seen in hCG reponsiveness, Leydig cells from the cryptorchid testis also show increased responsiveness to LHRH agonists *in vitro*, as 'testicular LHRH' has been implicated in local control processes(see below).

The paradox that remains is how can these local mechanisms explain the differences between *in vivo* and *in vitro* responsiveness of Leydig cells? It may be explained by the observation that testicular blood flow is reduced in the cryptorchid testis(Wang, Galil & Setchell, 1983), which would lead to a decreased exposure of

Leydig cells to LH. The raised serum LH levels found in bilaterally cryptorchid rats(Risbridger, Kerr & de Kretser, 1981c) may be one way in which the animal then ensures that adequate LH levels exist within the testis. Of interest also is the increased capability of Leydig cells to bind oestradiol in cryptorchid testes.

Oestradiol receptors have been shown to increase dramatically following cryptorchidism, doubling within 7 days and reaching four times control levels 28 days post-operation(Keel & Abney, 1980). Oestradiol concentrations within the testis are unchanged over 21 days, although there is a possible rise in oestradiol at 14 days, but oestradiol concentrations are significantly decreased by day 28 post-operation. Both LH and FSH receptors are reduced in the abdominal testes of unilaterally cryptorchid animals, although it is not clear how this occurs(Risbridger, Kerr & de Kretser, 1981c). Since loss of LH receptors precedes any rise in serum LH it is likely that either local damage or locally secreted factors produce these changes(Risbridger, Kerr & de Kretser, 1981c).

Recently, evidence on the restoration of testicular function following replacement of the cryptorchid testis within the scrotum (orchidopexy) has suggested that germ cells may also play a major role in intra-testicular communication(Jegou, Peake, Irby & de Kretser, 1984b). In adult rats even after only 10 days in the cryptorchid state, recovery of spermatogenesis following orchidopexy is very poor(Jegou, Laws & de Kretser, 1983a) and Leydig and Sertoli cell function remains impaired. Cryptorchidism induced in immature animals also impairs Sertoli and Leydig cell function in a similar way to that seen in the mature animal(Jegou et al, 1984b) but even after 21 days in the abdominal position orchidopexy can restore testicular function and

fertility. It is suggested that the cryptorchid position of the testis is not the major influence on Sertoli and Leydig cell function but rather that the presence of germ cells and the ability of the immature testis to replenish its germ cells plays a major role in regulating testis function. This data is in agreement with the concept of cyclical secretion of Sertoli cell products which may be dependent on specific Sertoli germ-cell interactions(see section 1.4.1).

However, despite the usefulness of surgically induced unilateral and bilateral cryptorchidism as an experimental model, this condition is not identical to the failure of testicular descent seen in man. It has been shown that whereas in the experimental replacement of testes within the abdomen(secondary cryptorchidism) the germ cells are the first to show signs of degeneration, in animals in which testicular descent is prevented(primary cryptorchidism) the Sertoli cells are those which are first damaged(Bergh, 1983a). Also in primary cryptorchidism there is evidence that rather than becoming hyperplasic, the Leydig cells are actually reduced in size(Bergh & Damber, 1978). Therefore whilst experimentally induced secondary cryptorchidism is of great value as an experimental model, care should be taken before extrapolating results obtained in such studies to cryptorchidism in man.

ii) Heat damage:

The aetiology of the impairment of spermatogenesis in cryptorchidism is unclear but it is believed to stem from damage due to the higher temperature to whcih testes are exposed in the abdomen. Reports of reversible impairment of testicular function following short term(15 minutes) heating of the testis in situ will therefore prove of great interest in the further assesment of such interactions.

It has been shown that heating the adult rat testis to 43 degrees centigrade will reduce specific germ cell populations, and that this is accompanied by reversible impairment of Sertoli and Leydig cell functions. It is not yet clear which of these changes occurs first, but this data provides further evidence for the close interdependence of these cell types(Jegou, Laws & de Kretser, 1984a). The relevance of these studies to human infertility can be seen when it is recognised that bathing for 15 minutes at temperatures in excess of 43°C is not uncommon, and a reported 'addiction' to such hot baths has indeed been shown to cause profound reductions in sperm numbers, motility and morphology(Eliasson & Virji, 1985).

iii) Efferent duct ligation:

Following experimental blocking of the efferent ducts of the rat testis by ligation, spermatogenesis becomes disrupted, with maximal damage occurring within three weeks, by which time the seminiferous tubules show marked signs of germ cell depletion and spermatogenic failure(Smith, 1962). More recently, these effects have been shown to be linked to morphological and functional changes in Leydig cells similar to those seen in cryptorchidism(Risbridger, Kerr, Peake & de Kretser, 1981a), suggesting that any damage of the seminiferous epithelium results in changes in the properties of Leydig cells. The fact that the changes in Leydig cell structure and function occur in the absence of any change in testicular temperature, is a further indication that while the Sertoli cell changes in cryptorchid or 'heated' testes may be a result of temperature damage, the changes which occur in the Leydig cells are almost certainly mediated by the Sertoli cells rather than being aspecific temperature effects. Vitamin A deficiency, hydroxyurea

treatment and foetal irradiation, all of which damage the seminiferous tubules, also produce focal hypertrophy of the Leydig cells, providing further evidence to support the role of the Sertoli cell in the regulation of the Leydig cell(Rich, Kerr & de Kretser, 1979).

iv) Anti-androgens:

In a study in which local damage within the testis was induced by implanting threads impregnated with anti-androgens there was massive hyperplasia of the interstitial tissue only in the localities immediately surrounding the damaged areas induced by this treatment(Aoki & Fawcett, 1978). It is argued that the most likely explanation for this effect is altered function of the damaged Sertoli cells. The effects on Leydig cell structure are nearly identical to those resulting from cryptorchidism etc;(as discussed above) and it therefore seems likely that any alteration of Sertoli cell function results in marked stimulation of adjacent Leydig cells(Sharpe & Rommerts, 1983).

It is thus obvious that Sertoli-Leydig cell functions are closely linked despite the functional barrier between them at the level of the peritubular cells(see above). Recently, evidence for a Leydig cell cycle linked to the spermatogenic cycle has been described(Bergh, 1982; 1984), in which Leydig cells adjacent to seminiferous tubules at stages VII-VIII have been shown to be significantly larger than those adjacent to either blood vessels or tubules at other stages of the spermatogenic cycle. This effect is abolished in the abdominal testis of unilaterally cryptorchid rats(see Bergh & Damber, 1984).

B) Effects of FSH treatment:

FSH has been shown to produce effects on the interstitial tissue despite the evidence which shows there are no FSH

receptors on Leydig cells(Purvis, Clausen & Hansson, 1979). Because of the contamination of all FSH preparations with small amounts of LH, it has been suggested that the observed 'FSH' effects are in fact due to contaminating LH(Purvis et al, 1979), or that FSH acts synergistically with small amounts of LH to produce these effects (Davies, 1981). During sexual maturation, testicular LH receptor numbers increase in parallel with the plasma levels of FSH, whilst LH levels remain low(Ketelslegers, Hetzel, Sherins & Catt, 1978). FSH also increases the LH-stimulated production of testosterone by adult Leydig cells in the rat(Aznar, Diaz, Herrera-Justiniano & Aznar, 1979), possibly by affecting 17β-hydroxysteroid dehydrogenase activity, an enzyme also affected by FSH in immature rats(Welsh & Wiebe, 1978). FSH also increases the activity of both 5α-reductase and 3β-hydroxysteroid dehydrogenase in immature rats(Davies, 1981), whilst effects on the conversion of progesterone to testosterone have also been reported in hypophysectomized adult rats(Sivelle, 1979). More recently, studies in hypophysectomized immature rats have yielded strong morphological and biochemical evidence for the involvement of FSH in the maturation and proliferation of Leydig cells(Kerr & Sharpe, 1985a,b), and have suggested that such effects are mediated via the seminiferous tubules.

Although indirect, this data argues strongly for the presence of an intricate system of communication between the Sertoli cell within the seminiferous tubule and the Leydig cell in the interstitial space of the testis. However, none of the studies mentioned in the above 2 sections(A & B) have identified the humoral agent(s) involved in the changes described. A number of possible candidates have, however, been identifed in recent functional studies.

C) Functional Evidence:

Despite the widely documented diversity of Sertoli cell products, up to this point in time only two locally produced testicular hormones have been shown definitively to exert a direct effect on the Leydig cell; testicular 'LHRH' and oestradiol.

i) Testicular 'LHRH':

Testicular function and development in mammals is primarily controlled by the pituitary gonadotrophins, which in turn are under the control of the hypothalamic decapeptide Luteinizing Hormone Releasing Hormone(LHRH), also know as GnRH, (gonadotrophin releasing hormone). LHRH is released in a pulsatile fashion into the hypothalamic pituitary portal vessels and acts directly on the anterior pituitary to stimulate release of LH and FSH(Kalra & Kalra, 1983; Clayton & Catt, 1981; Conn, Marian, McMillian, Stern, Rogers, Penna & Grant, 1981).

The theory that LHRH might have functions in tissues other than the pituitary conflicted with one of the central dogmas of reproductive physiology. However LHRH-like peptides have been found to be present in a wide range of tissues, including the adrenals, the pancreas, the pineal gland, placenta, ovary and testis(Sharpe, 1984). In addition chronic treatment in vivo with LHRH analogues has a wide range of effects on testicular function(Hsueh & Jones, 1981), resulting in the impairment of many testicular parameters. Chronic LHRH treatment has been shown to decrease testicular androgen production in both adult and immature rats(Bambino, Schrieber & Hsueh, 1980; Huhtaniemi, Stewart, Channabasavaiah, Fraser & Clayton, 1984a,b; Lacroix, Eechaute & Leusen, 1984), and to decrease testicular LH and prolactin receptor numbers(Bambino, Schrieber & Hsueh, 1980; Catt,

Baukal, Davies & Dufau, 1979; Auclair, Kelly, Coy, Schally & Labrie, 1977, Huhtaniemi et al, 1984a,b; Marchetti & Labrie, 1984). Testis weights, along with seminal vesicle and prostate weights were reduced following LHRH treatment(Cusan, Auclair, Belanger, Ferland, Kelly, Seguin & Labrie, 1979; Huhtaniemi et al, 1984a,b) and as might be expected in the light of these changes spermatogenesis was greatly impaired(Heber & Swerdloff, 1981; Pelletier, Cusan, Auclair, Kelly, Desy & Labrie, 1978). However the potential of LHRH as a male contraceptive is marred by its effects on testicular weight and androgen production, as well as by reports suggesting that even following 12 weeks of treatment in rats, and despite the above changes, fertility is still maintained in about half of the treated group(Vickery, McRae, Bergstrom, Briones, Worden & Seidenberg, 1983). Although these results strongly suggest an intratesticular role for LHRH, it is possible that they are mediated wholly or in part via the action of this hormone on pituitary secretion of gonadotrophins.

Studies in hypophysectomized rats have shown that comparable effects of LHRH agonists also occur in the absence of the pituitary gland, although the down regulation of LH receptors is not as marked (Hsueh & Jones, 1981; Lacroix, Eechaute & Leusen, 1984; Marchetti & Labrie, 1984), suggesting that the effects seen in intact rats are at least partly mediated via increased pituitary secretion of LH. In experiments in which an LHRH agonist was injected intratesticularly into one testis only, a significant increase in testosterone levels in interstitial fluid from the treated but not from the contralateral control testis was noted(Sharpe, Doogan & Cooper, 1983a). These effects could not be attributed to peripheral gonadotrophins since an increase in these hormones would affect both

testes. In animals treated with higher doses of LHRH agonist significant increases in total testis testosterone and spermatic vein testosterone levels were also seen in the treated when compared with the control testes(Sharpe, Doogan & Cooper, 1983a). Interstitial fluid formation was also modulated by LHRH agonist treatment, there being a significant reduction in interstitial fluid levels(Sharpe, Doogan & Cooper, 1983a). This latter result is of interest, since if it is assumed that the interstitial fluid volume reflects the permeability of testicular capillaries and therefore access of LH to the testis it can be seen that LHRH agonist increased testicular testosterone production whilst decreasing testicular capillary permeability and hence testicular exposure to peripheral LH.

LHRH receptors within the testis are located only on the Leydig cells(Sharpe, 1982; Clayton & Catt, 1981) and have an affinity similar to that of both ovarian and pituitary LHRH receptors(Clayton & Catt, 1981; Dalkin, Bourne, Pieper, Regiani & Marshall, 1981). In the male rat LHRH binding in gonadal tissues is apparent in animals of all ages from day 1 onwards, and increases gradually with age(Huhtaniemi, Catt & Clayton, 1985) although earlier reports failed to show binding in animals below 40 days of age(Dalkin et al, 1981). The testicular concentration of LHRH receptors varies biphasically, being highest at day 1 post-partum(3.2+/-0.4 fmoles/g tissue, mean+/-se), declining to a nadir at day 15 post partum(1.5+/-0.5 fmoles/g tissue) and thereafter increasing with age during the period investigated(up to 60 days post partum, 2.7+/-0.3 fmoles/g tissue), this binding pattern reflecting closely the volume density of Leydig cells at these times(Huhtaniemi et al, 1985). Administration of an LHRH agonist increased testosterone production in testes of all ages during these

studies(Huhtaniemi et al, 1985) showing that the LHRH receptors demonstrated are functionally active. The Ka of the receptors reported in this study(Huhtaniemi et al, 1985; 1.8 x $10^9 M^{-1}$) agrees with that reported by Dalkin et al(1981; 6.5x10^9 M^{-1}) however binding levels reported by Dalkin et al(1981) are much higher than those reported above with binding levels at 60 days of about 160fm/mg protein from interstitial tissue(Dalkin et al, 1981). Although, the dissimilarities between the tissues used(whole testis vs interstitial tissue) makes comparison of receptor numbers between these studies difficult.

An 'LHRH-like' peptide has been reported within the testis (Sharpe, 1982; 1983; Sharpe & Harmar, 1983; Bhasin, Heber, Peterson & Swerdloff, 1983) which presumably interacts with Leydig cell LHRH-receptors(Sharpe, 1983; Sharpe & Fraser, 1980b). Its activity may be modulated by hCG(Sharpe & Fraser, 1980a) and it appears to be immunologically dissimilar to hypothalamic LHRH(Bhasin et al, 1983; Sharpe & Harmar, 1983; Sharpe, 1982). The Sertoli cell has been suggested as a likely source for this factor, since Sertoli cell culture medium contains LHRH-like activity(see Sharpe 1984). Testicular 'LHRH' has so far been purified to a very limited extent and there may be more than one active component(Bhasin et al, 1983). Although there are testicular receptors for testicular 'LHRH', its physiological significance is unknown, and in this context the extremely low amounts of testicular 'LHRH' detected(less than 50pg LHRH agonist equivalents per testis) and the detection of similar levels in liver tissue leave its' physiological significance open to question(Sharpe & Harmar, 1983).

Studies on LHRH effects in the testis have been limited by the

failure to purify the active testicular 'LHRH' and this has led to the use of synthetic hypothalamic LHRH and its analogues instead. Despite early reports that LHRH had no effect on the Leydig cell in vitro (Hsueh & Jones, 1981; Sharpe, 1982; Badger, Beitins, Ostrea, Crisafulli, Little & Saidel, 1980) it is now accepted that while LHRH has no effects on the Sertoli cell(Gore-Langton, Lacroix, Dorrington, 1981), its effects on the Leydig cell vary widely with dosage and time of incubation. Although reports prior to 1982 suggested that the effect of LHRH on the testis and therefore the Leydig cell was to impair testosterone production the majority of these reports involved chronic treatment with LHRH analogues, and more recent studies have shown that the initial effect of LHRH on the Leydig cell both in vitro and in vivo is to stimulate testosterone production(Hunter, Sullivan, Dix, Aldred & Cooke, 1982; Sharpe, Doogan & Cooper, 1982). This stimulatory effect shows a 1-2 hour lag phase and studies suggest that the Leydig cells need to be exposed continously to LHRH agonist if the stimulatory effect is to be maintained, since preincubation with LHRH agonists produced no subsequent stimulation of testosterone production (Sharpe & Cooper, 1982 a,b). LHRH has been shown to stimulate phosphatidylinositol metabolism in rat granulosa cells and more recently to stimulate phosphatidylinositol and prostaglandin E production in Leydig cells(Davies, Farese & Clark, 1983; Molcho, Zakut & Naor, 1984), and these effects may indicate the intracellular mechanism of action of LHRH after binding to its receptors. In trying to discern why there is such a difference between acute and chronic LHRH effects on steroidogenesis it has been shown that both the duration of exposure of Leydig cells to LHRH and the co-incident exposure of these cells to LH affect their responses to LHRH(Sharpe, Doogan & Cooper,

1983b; Sharpe & Fraser, 1983). Treatment of rats with an LH-antiserum has been shown to markedly increase Leydig cell LHRH receptors and responsiveness to LHRH agonists suggesting that LH negatively regulates LHRH receptor numbers and responsiveness(Sharpe & Fraser, 1983).

The ability of LHRH to modulate the testicular response to LH stimulation, its ability to alter capillary permeability and also the possibility that it may act over short time periods all suggest that testicular LHRH may be an important local regulator of testosterone production in the testis.

ii) Oestradiol:

The role of oestradiol in the regulation of testicular steroidogenesis is still unclear. Early investigations involving this hormone led to suggestions that it might be a major regulator of testosterone production in the testis. The suggestions were based on three areas of research: (1) The recognition and characterization of Leydig cell oestradiol receptors. (2) The characterization of Leydig cell response to oestradiol and oestrogen treatment, and (3) The discovery that Sertoli cells produce oestradiol *in vivo* and *in vitro* (See Sharpe, 1982, for review).

The background to these investigations provides an illustration of the pitfalls of applying *in vitro* results too readily to the *in vivo* situation. The presence of testicular oestradiol receptors and their physical characteristics have been established in the rat(see Mulder, van Beurdan-Lamers, de Boer, Brinkman & van der Molen, 1974, for review). The interstitial cells of the rat testis have been shown to contain two types of binding sites for oestradiol, a high affinity, low capacity site and a low affinity, high capacity site. The high

affinity receptor has been partially purified and identified as a heat sensitive protein which is translocated to the nucleus of the Leydig cells. No significant levels of this receptor were found in seminiferous tubules. The association constant for this receptor class was $10^{10}M^{-1}$, with a concentration of $2x10^{-14}$ moles/mg cytosol protein(Mulder et al, 1974).

Oestradiol treatment of hypophysectomized rats decreased the Leydig cell response to LH in vitro without altering hCG binding(Damber, Bergh, Daelin, Ekholm, Selstam & Sodergard, 1983), and whilst injected oestrogens have been shown to exert inhibitory effects on testosterone production in rats and men, there is some dispute as to whether these results are the consequence of a reduction in serum LH levels, or represent a direct effect of the oestrogens on the testis, or a combination of both effects(See Sharpe, 1982). The most acceptable explanation for these effects is that oestrogens affect both the testicular response to LH and serum levels of this hormone(see Sharpe, 1982 for review).

The final set of experimental data supporting the role of oestradiol as an intratesticular regulating factor was the detailed evidence that the Sertoli cell produces oestradiol in response to FSH stimulation (see Sharpe, 1982 for review). Oestradiol production by the Sertoli cell occurs via the conversion of androgens to oestrogens, and it has been shown that FSH can regulate the conversion of 19-hydroxylated androgens to oestrogens(Dorrington, Fritz & Armstrong, 1976). The above evidence, taken in isolation, strongly suggested a regulatory role of oestrogens produced by the Sertoli cell on Leydig cell testosterone production, and also that Leydig cell testosterone forms the substrate for the Sertoli cell production of oestrogens, i.e. a

two-cell theory of oestrogen synthesis as occurs in the ovary. However, more recent evidence has shown that this is not the case. In vivo, Sertoli cells are now known to secrete oestradiol only up to about day 20 of life, after which their ability to produce oestrogens rapidly disappears(Pomerantz, 1979; Rommerts, de Jong, Brinkman & van der Molen, 1982). In addition, studies on the ontogeny of Leydig cell oestrogen receptors show that these appear only after day 14 or as late as day 21 of life(de Boer, Mulder & van der Molen, 1976; Abney & Melner, 1979), and are paralleled by the appearance of adult Leydig cells which synthesise oestradiol(see above). In adult animals FSH produces no effect on testicular oestradiol levels or aromatase activity, while hCG rapidly increases testicular oestradiol levels and aromatase activity(see Sharpe, 1982 for review).

Therefore during the period when Sertoli cells are actively converting testosterone to oestradiol, the Leydig cells contain no receptors for this steroid, and once the adult Leydig cells appear production of oestradiol switches from the Sertoli cell to the Leydig cell, thus effectively ruling out any role oestradiol has as a link between the seminiferous tubules and the interstitium.

However, the effect of oestrogens on the Leydig cell continued to generate interest, both as to their role in the regulation of this cell type and as to their physiological significance. It has been suggested that oestradiol acts as an ultra-short loop feedback regulator of testosterone production(Catt, Harwood, Clayton, Davies, Chan, Katikineni, Nozu & Dufau, 1980), but a number of factors raise doubts as to the physiological significance of such a feedback. Firstly, only levels of oestrogens far in excess of those seen in vivo have been shown to effectively inhibit testosterone production in

vitro. However, it can be argued that, since oestradiol is produced within the Leydig cell, such apparently supra-physiological levels may exist within the cell(see Sharpe, 1982 for review). Secondly, and more recently, it has been suggested that the effect of oestradiol on the Leydig cell is not in fact mediated via the Leydig cell oestrogen receptors, since the effects of oestrogens were not blocked by tamoxifen, a potent competitive inhibitor of oestrogen action (Damber et al, 1983).

Although oestradiol was once thought of as an intratesticular messenger with a clearly defined regulatory function, it is now unclear what its function is in physiological states, although it is certain that it is not as was once suggested an intratesticular messenger between Sertoli and Leydig cells.

1.4.6: Other potential regulators of testicular function:

There is now evidence for the existence of an increasing number of factors of intratesticular origin, each of which may play a role in the regulation of testicular function. These factors may not, as yet, have specifically defined actions, and often their sites of production are unknown. The list of factors that follows is by no means exhaustive, and there is no specific order of precedence but the range of factors represented will no doubt give some idea of the immensity of the task that faces those investigating cellular communication within the testis and the degree of research required before a clear picture will emerge.

i) Gonadotrophin receptor binding inhibitors:

Reports of factors produced within the testis which specifically inhibit FSH and LH binding to their testicular receptors are becoming more numerous. As yet the physiological

significance of these factors is not known, although if they do represent true intratesticular factors their potential for modulating testicular function is obviously of great interest. Two types of binding inhibitors have been reported, those which inhibit FSH binding and those which inhibit LH binding, and this division of function into two distinct sub-classes encourages the belief that these factors may be of physiological significance.

a) FSH binding inhibitors:

Early experiments with testicular membrane preparations led to the suggestion that these preparations contained an FSH binding inhibitor(Abou-Issa & Reichert, 1976). Further studies showed the presence of a small molecular weight inhibitor of FSH binding(Reichert & Abou-Issa, 1977). This factor has a molecular weight of about 1500 daltons and is present in testicular extracts. The significance of these findings is called into question however, by the presence of a similar factor present in kidney and liver extracts(Reichert & Abou-Issa, 1977) and its presence in serum is also unexplained(Reichert, Sanzo & Darga, 1979). The serum factor has a molecular weight of about 700 daltons, and is heat stable over short periods, although it is susceptible to peptidases, which suggests a peptide structure(Reichert, Sanzo & Darga, 1979; Dias, Treble, Bennet & Reichert, 1981). FSH binding inhibitors have also been reported in human seminal plasma, where two different species of inhibitor are found, a low molecular weight peptide of about 1,000 daltons, and a high molecular weight peptide of about 19,500 daltons(Dias et al, 1981). A factor with comparable effects has also been identified in bovine testis and has a molecular weight of around 30,000 daltons(Dias & Reichert, 1983). Research on FSH binding inhibitors

in bovine follicular fluid shows a correlation between the level of inhibitor and the mean atretic rank of the follicles, suggesting that the levels of inhibitor may be linked to the physiological condition of the follicles from which it is isolated (Sluss, Fletcher & Reichert, 1983). Correlation of increased testicular levels of inhibitors with increasing age in rats further supports a physiological function, still unknown, for these inhibitors. However, it should be noted that levels of binding inhibitor in liver and kidney also show an age related increase(O'Shaughnessy, 1979).

Despite evidence for the existence of FSH binding inhibitors in a number of species, there is as yet no clear evidence that these factors are either tissue specific or modulated by physiological endocrine events. It has been suggested that since the gonads are the only site of FSH binding then demonstration of extragonadal production of FSH binding inhibitors is not necessarily relevant to their physiological role. Notwithstanding this, there is as yet no clearly defined role for such an inhibitor *in vivo*.

b) LH binding inhibitors:

The evidence for LH binding inhibitors presents a similar story to that for FSH, stemming from initial reports of anti-LH activity in the gonads of various species(Yang, Samaan & Ward, 1976b). A heat resistant peptide with a molecular weight of about 3,800 daltons was isolated from ovaries of pregnant or pseudopregnant rats and this peptide inhibited LH binding to ovarian LH receptors, and evidence suggested that two forms of this peptide existed within the ovary (Yang, Samaan & Ward, 1976b). No activity was detected in testis or liver extracts nor in ovaries of non-pregnant or immature

rats(Yang, Samaan & Ward, 1976b). Further studies showed that after freezing ovaries from normal rats for up to 10 weeks, LH binding inhibitors could be detected. This inhibitor appeared to be specific for the ovarian LH receptor, having little or no effect on LH binding to testicular LH receptors(Yang, Samaan & Ward, 1979). The inhibition of LH binding in ovarian tissue resulted in decreased progesterone synthesis, although basal synthesis of progesterone was unaffected(Yang, Samaan & Ward, 1976a). The fact that other tissues, including testes, heart and liver, plus ovaries from immature rats, did not show any activity suggests that this factor may well have a physiological role(Yang, Samaan & Ward, 1976a). The peptide actually stimulated testicular testosterone production, but since the ovarian extract used was crude it is possible that other factors may have interfered in the test system, although a direct stimulatory effect of the ovarian LH binding inhibitor on testicular steroidogenesis cannot be ruled out(Yang, Samaan & Ward, 1976a).

More recently the presence of an LH binding inhibitor in rat testicular tissue has been reported(Rojas, Moretti-Rojas, de Bellabarba & Bishop, 1981). This factor, which has a molecular weight below 12,000 daltons and is partially heat labile, inhibits hCG binding to both ovarian and testicular LH receptors(Rojas et al, 1981), by competing for binding sites. Like the FSH binding inhibitor, this factor is not tissue specific and is present in other, non-gonadal, tissues, including kidney, liver, and brain(Rojas et al, 1981) and such findings cast doubt on its physiological significance.

ii) Pro-opiomelanocortin derived hormones:

Evidence for the involvement of peptides derived from the pro-opiomelanocortin(POMC) molecule in the control of testicular

function is growing rapidly, following the discovery of several of these peptides in the testis. However, as yet, data is sparse and little is known of the ways in which these peptides might influence testicular function.

Early evidence for opiate effects on steroidogenesis *in vivo* appeared to be mediated via depression of circulating LH levels, suggesting that the effects of these compounds were manifested at the hypothalamic or pituitary levels(Cicero, Meyer, Bell & Koch, 1976). More recently the production of β-endorphin, α-MSH and ACTH by Leydig cells of the rat, and its presence in other sites in the reproductive tract have centred attention on possible paracrine functions of the POMC-derived peptides(Tsong, Phillips, Halmi, Liotta, Margioris, Bardin & Krieger, 1982). While β-endorphin seems to be the major endorphin component present, α and γ-endorphins are also present, along with α-MSH(Margioris, Liotta, Vaudry, Bardin & Krieger, 1983). Reports demonstrate that the major site of synthesis of these peptides is the Leydig cell(Margioris *et al*, 1983; Shaha, Liotta, Krieger & Bardin, 1984; Chen, Mather, Morris & Bardin, 1984). Recent reports suggest that spermatagonia and spermatocytes contain a form of α-N-acetyl-endorphin, an inactive metabolite of β-endorphin, but that β-endorphin activity is confined to Leydig cells(Cheng, Clements, Lolait & Funder, 1984).

The pro-opiomelanocortin gene is not found in Sertoli cell lines, but is evident in two different Leydig cell lines(Chen, Mather, Morris & Bardin, 1984). Whilst there is evidence for synthesis of endorphins of different classes in the Leydig cells, there is no evidence for Sertoli cell production of endorphins or related compounds(Shaha *et al*, 1984; Margioris *et al*, 1983; Chen *et al*,

1984; Cheng et al 1984). Testicular interstitial fluid, an essential link in the communication between the seminiferous tubules and the interstitium, has been shown to contain β-endorphin and ACTH when collected from adult rats and levels of these hormones are altered following treatment with an analogue of LHRH(Valenca & Negro-Vilar, 1984). Opiate receptors have been identified in the testis(Boitani, Shaha, Bardin & Hahn, 1984) although as yet their location is not known. There is some evidence that testicular production of endorphins can be modulated by hormones. hCG stimulation increases the number of mouse interstitial cells staining positively for β-endorphin, and also increases β-endorphin levels in ovarian interstitial cells(Shaha et al, 1984; Rothrock, Sarkar & Erickson, 1984). Expression of the POMC-gene in granulosa cells is increased by concomitant stimulation with LH and FSH when compared to FSH stimulation alone(Melner, Luo, Roos, Clark, Roberts & Puett, 1984).

Preliminary evidence for a role of endorphins in the testis comes from a report showing increased Sertoli cell division in foetal rats following administration of naloxone, an antagonist of β-endorphin, suggesting that β-endorphin might inhibit Sertoli cell division (Orth, 1984).

Overall the evidence for a paracrine function of endorphins is growing rapidly, although there is as yet no conclusive evidence that the endorphins are involved in the physiological control of testicular function. Whilst endorphins are produced by the Leydig cells no receptors have been found in the Sertoli cells as yet, and although an effect of an endorphin antagonist has been demonstrated in foetal rats, there is currently no evidence for effects in the adult.

iii) Epidermal growth factor:

Epidermal growth factor(EGF) is a known mitogenic peptide found in serum(Hsueh, Welsh & Jones, 1981), and it has been shown to stimulate mitosis in cell lines which are thought to be derived from Sertoli and Leydig cells(Mather, 1980). However, more recent evidence suggests that EGF has a direct effect on Leydig cell steroidogenesis(Hsueh, Welsh & Jones, 1981; Welsh & Hsueh, 1982; St-Arnaud, Walker, Kelly & Labrie, 1983), having no effect on basal testosterone production in vitro but markedly inhibiting hCG stimulated testosterone production(Hsueh, Welsh & Jones, 1981). Comparable effects of EGF have been demonstrated on ovarian oestrogen production(Hsueh, Welsh & Jones, 1981). EGF has been shown to act via specific, high affinity, low capacity receptors on the Leydig cells (Welsh & Hsueh, 1982) and to act only after a 12 hour lag period. Decreased testosterone production under these conditions is linked with increased cellular protein concentrations, but no change in cellular DNA content is seen(Welsh & Hsueh, 1982). Since the presence of receptors for EGF and a specific effect of this peptide have been shown within the gonads it is possible that this peptide exerts a paracrine role. It may be that EGF is produced locally within the testis to modulate steroidogenesis, although the time taken for an effect to be manifested is lengthy which leaves the physiological significance of such effects open to question.

iv) Oxytocin, Vasopressin and Neurophysin:

Testicular material from both rats and man has been shown to contain oxytocin, vasopressin and the carrier proteins for these two compounds, neurophysin(See Wathes, 1984 for review). Oxytocin has been recently demonstrated to modulate rat seminiferous tubule

contractility(Wathes, 1984), and both arginine vasopressin and oxytocin have been shown to be potent inhibitors of hCG stimulated testosterone production by isolated Leydig cells(Wathes, 1984; Adashi & Hsueh, 1981a,b). Of nine compounds tested three(arginine-vasopressin, arginine-vasotocin and lysine-vasopressin) were highly active in inhibiting hCG-stimulated testosterone production, the minimum effective dosage for all of these compounds being around 10^{-10}M, while mesotocin, valitocin and oxytocin were of intermediate efficacy with minimum effective doses of around 10^{-8}M. Three compounds (isotocin, glumitocin and aspartocin) were almost ineffective at doses of 10^{-4}M(Adashi & Hsueh, 1981a).

Further studies have shown that the effects of argine-vasopressin and oxytocin are mediated by specific testicular recognition sites, which are susceptible to agonism and antagonism by compounds related to vasopressin and oxytocin(Adashi & Hsueh, 1981b).

The fact that vasopressin and oxytocin can completely reverse the hCG stimulated increase in Leydig cell testosterone production(Adashi & Hsueh, 1981a,b) and that these hormones are present in testicular tissue suggests a local role for these compounds in the regulation of steroidogenesis. However since levels of oxytocin and vasopressin are low in testicular tissues, and other roles of these hormones are more convincing physiologically, e.g. in modulating contractitily of seminiferous tubules, then it may be premature to assume that they regulate steroidogenic function in the physiological state.

v) Prostaglandins:

The role of prostaglandins in the regulation of testicular function is unclear, as conflicting reports of stimulatory and

inhibitory effects of prostaglandins on Leydig cell function *in vitro* are found in the literature(see Sharpe, 1982 for review). However the presence on the Leydig cell of specific high affinity receptors for PGE, and demonstrable increases in cAMP production following prostaglandin binding to such receptors makes it possible that such molecules are involved in the regulation of steroidogenesis(Sebokova & Kolena, 1978). Macrophages from a number of tissue sources and species have been shown to produce prostaglandins(Hume & Gorden, 1982), and recent reports suggest that macrophages may interact with Leydig cells within the testis(Bergh, 1985; see also Chapter 8). However, as yet the evidence is incomplete and further investigations are required before such possibilities can be confirmed or refuted.

vi) Inhibin:

Although the major role of inhibin is the selective regulation of pituitary FSH secretion, recent evidence suggests that it may have local effects within the testis. Inhibin preparations have been shown to inhibit the incorporation of labelled thymidine into testicular, but not liver, DNA *in vivo* and also *in vitro*(Franchimont, Croze, Demoulin, Bologne & Hustin, 1981). These results are similar to effects obtained with testicular chalones and it has been postulated that inhibin may be identical to these compounds(Franchimont *et al*, 1981). Another postulated local effect of inhibin is the modification of basal and FSH stimulated cAMP-phosphodiesterase activity(Sheth, Vijayalakshmi, Sheth, Bandivdekar & Moodbridi, 1982), with inhibin increasing cAMP-phosphodiesterase activity basally and in the presence of FSH in both immature and adult rats(Sheth *et al*, 1982). It has also been suggested that inhibin could interfere with FSH binding to its

receptors within the testis(Vijayalakshmi, Moodbidri, Bandivdekar & Sheth, 1980). However, these and other inhibin studies have been challenged since the investigators do not as yet have access to purified inhibin, and therefore, while these results are of interest, the elucidation of the true physiological effects, if any, of inhibin within the testis must await the synthesis or purification of the active peptide.

vii) Thyroid releasing hormone(TRH):

Since TRH was first identified in the rat prostate(Pekary, Sharp, Briggs, Carlson & Hershman, 1983), investigations as to its origin have been in progress. The presence of TRH in human and rat prostates, and human semen and testis has been shown, along with high levels of TRH degrading enzymes in Sertoli cells(Pekary, Ross, deKernion & Hershman, 1983; Pekary & Rosen, 1982). Interstitial cells have been shown to be the source of this peptide(Pekary & Rosen, 1982), and it has also been suggested that testosterone regulates the testicular production of TRH(see Pekary, Ross, deKernion & Hershman, 1983) Therefore, the function of this peptide within the reproductive tract is not clear.

viii) Testicular-anti-mullerian hormone:

Anti-mullerian hormone controls the regression of the Mullerian ducts in male foetuses, and has been characterized as a polymer of two or three 72,000 dalton monomers(Picard & Josso, 1984). This hormone has recently been isolated from rete testis fluid from adult bulls(Josso, Picard, Dacheux & Courot, 1979), and, as it is known to derive from the Sertoli cells(Vigier, Picard, Tran, Legeai & Josso, 1984), it represents yet another potential regulator of the testis, although its function is not yet known.

ix) Renin-angiotensin:

It has recently been demonstrated that angiotensin and its precursor renin are present in Leydig cells(Pandey, Melner, Misono & Inagami, 1984). Rat Leydig cells from two distinct populations separated on metrizamide gradients had different levels of angiotensin I and also of the inactive precursor renin(Pandey, Melner, Misono & Inagami, 1984). Angiotensins II and III were also present. When compared with kidney renin, rat testicular renin had a similar molecular weight and immunoreactivity(Parmentier, Inagami & Pochet, 1984). The significance of these findings is still unclear.

x) Corticotrophin releasing factor:

Specific high affinity binding sites for corticotrophin releasing factor have been reported in a range of rat tissues including spleen, liver, and testis(Dave, Bisserbe & Eskay, 1984). Their significance is not known.

xi) Clusterin:

Recently a polypeptide capable of inducing clustering in cultures of various cell types has been identified in testes and ovaries, but not in a range of other tissues(Fritz, Blaschuk & Burdzy, 1984). Clusterin is a heat-stable, trypsin-sensitive peptide and is effective in aggregating Sertoli and other cell types *in vitro*. Its actions *in vivo* are unknown(Fritz, Burdzy, Setchell & Blaschuk, 1983).

CHAPTER 2

MATERIALS & METHODS

2.1: Animals and treatments:

2.1.1: Animals:

All the animals used for these studies were Sprague Dawley male rats, either from our own colony or obtained from Charles River Ltd(U.K.). Rats from our own colony were housed under conventional conditions(Light:dark cycle comprising 14h light:10h dark, food and water ad libitum, temperature 21°C). All rats obtained from Charles River were subsequently housed under these conditions. All animals were killed using dry ice generated carbon dioxide and cervical dislocation. In the course of these studies the following treatments were used:

2.1.2: Cryptorchidism:

During the course of these studies a number of rats were subjected to either unilateral or bilateral cryptorchidism. Operations were carried out under ether anaesthesia. The testes were exposed and either one(unilaterally cryptochid) or both(bilaterally cryptorchid) were translocated into the abdominal cavity. Subsequently the inguinal canal was closed by sutures to prevent the redescent of the testes. I am grateful to Mr Denis Doogan for performing all such operations required for these studies.

2.1.3: Hypophysectomy:

Male Sprague-Dawley rats, which had had their pituitaries removed via the parapharyngeal route, were obtained from Charles River. In addition to the normal diet given to our own colony, hypophysectomized rats received 5% dextrose in their drinking water.

2.1.4: Injections:

i) LH: Groups of rats were injected with either 0.7mls 0.9% (w/v) sodium chloride(controls) or 10 μg ovine LH(NIH-LH-S24),

subcutaneously.

ii) LH antiserum: Groups of adult rats 80-85 days of age were injected subcutaneously with an LH antiserum as a single dose of 0.7 mls and animals were killed at intervals between 2-40 h later. The LH antiserum was raised in rabbits and was a gift from Dr. H.M. Fraser; its properties have been described by Sharpe & Fraser(1983). Control rats were injected with an equivalent volume of 0.9% sodium chloride.

iii) Ethane dimethanesulphonate(EDS): Groups of adult rats were injected intraperitoneally with either 75mg EDS/kg body weight in dimethyl-sulphoxide-water(1:3 v/v) or with an equivalent volume of vehicle.

2.2: Preparation of Leydig cells:

2.2.1: Interstitial cell preparation:

Testes from adult(70-90 day old) rats were removed immediately following death, decapsulated and placed in 50 ml conical flasks in groups of 4-5 testes per flask. Collagenase(0.25 mg/ml; Sigma, U.K. Type I) and bovine serum albumin(2.5 mg/ml; BSA, Fraction V, Sigma U.K.) were added to M199 containing Hank's salts and 20mM Hepes buffer (M199H, Flow Laboratories, U.K.). 1.5 mls/testis of this solution were added to the flasks and the testes dispersed for 6-7 minutes in a shaking water bath(170 cycles/minute, 32^{o}C). After dispersion groups of 8-10 testes were decanted into 50 ml measuring cylinders, diluted with 50 mls of M199H and mixed by 2-3 gentle inversions of the cylinder. The tubular mass was then allowed to settle under gravity and the supernatant, containing the interstitial cells, decanted into 5 ml plastic centrifuge tubes. The tissue mass was then discarded. The interstitial cells were recovered by centrifugation at 4^{o}C for 5 min

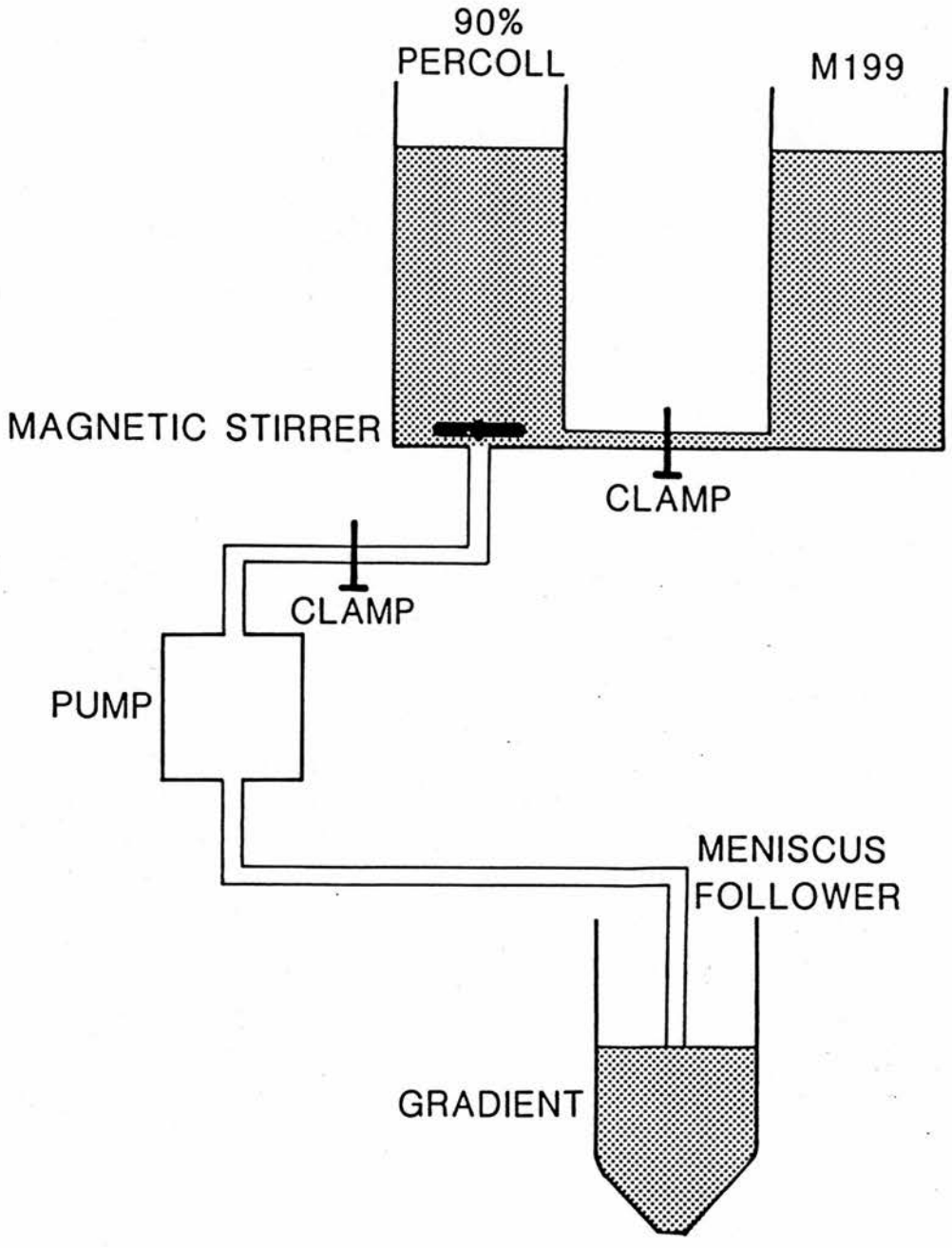

Fig 2.1: Apparatus for formation of continuous Percoll gradients: By placing 90% Percoll in the left hand chamber and M199H in the right hand chamber continuous gradients are formed by the mixing of these two chambers throughout the pumping procedure.

at 250 x g, on a Mistral 2L centrifuge(Beckman Ltd). The supernatant was decanted and the tubes blotted dry prior to resuspension of the precipitated cells in 6 mls of M199H. Cells were then stored at 4°C prior to further purification(see below) or for use as a "crude" Leydig cell preparation. Cells isolated in this manner usually contained 15-25% Leydig cells as identified by 3βHydroxysteroid dehydrogenase staining of air-dried smears(Sharpe & Fraser, 1983).

2.2.2: Percoll purification of Leydig cells:

a) Formation of Percoll Gradients:

i) Preparation of stock solutions:

Percoll(Pharmacia Ltd. U.K.) is a medium composed of colloidal silica coated with polyvinyl-pyrrolidone to reduce toxicity. It has a low osmolality(20 mOsmoles/kg H_2O) and must therefore be adjusted to form an isotonic solution, i.e. a solution which is normo-osmotic. Stock Percoll was mixed with 10 times normal strength M199H (9:1 v/v, Flow Laboratories) producing a solution containing 90% Percoll and normal concentrations of M199H. This solution, hereafter termed '90% Percoll', has a density of 1.12 g/ml, and it was then diluted further with normal strength M199H to form continuous or discontinuous gradients.

ii) Formation of continuous Percoll gradients:

For each 50 ml continuous gradient 25 mls of M199H and 25 mls of 90% Percoll were prepared. The 90% Percoll was placed in the left-hand chamber of a gradient former(Fig 2.1) and the M199H in the right hand chamber. Using a magnetic stirrer to mix these solutions 0-90% Percoll gradients were prepared using a Densi-Flow combined pump and meniscus follower(Searle & Co, U.S.A.), with densities ranging from 1.0-1.12 g/ml.

Density g/ml	Volume of 90% Percoll per 10 ml fraction (mls)	Volume of M199H per 10 ml fraction (mls)
1.00	0.00	10.0
1.03	2.56	7.44
1.05	4.27	5.73
1.07	5.98	4.02
1.09	7.69	2.31

Table 2.1: Formation of discontinous Percoll gradients: Volume of 90% Percoll and of M199H required for preparation of the fractions of differing densities for the formation of discontinous Percoll gradients.

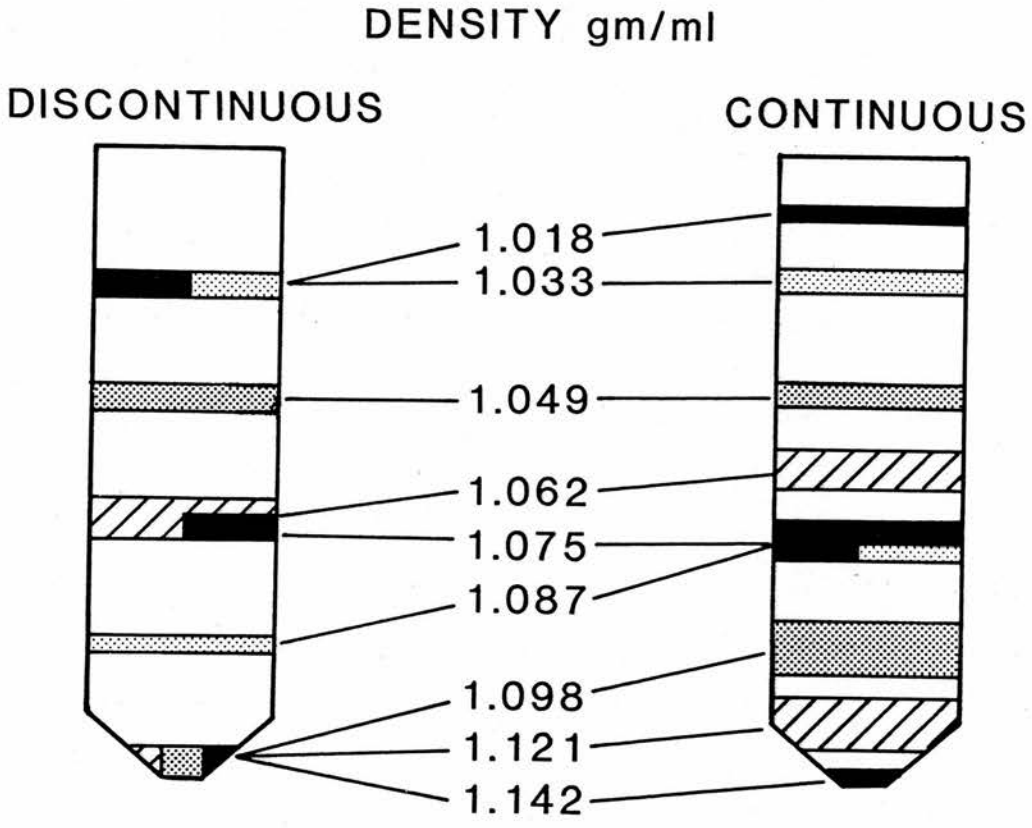

Fig 2.2: Comparison of continous and discontinuous Percoll gradients using Density Marker Beads(DMB, Pharmacia U.K.). It can be seen that whilst in the continous gradient a wider distribution of the DMB are seen, the bands in which they appear are also more diffuse than those seen on the discontinous gradient. On the discontinous gradient the DMB form five distinct and tight bands.

iii) Formation of discontinous Percoll gradients:

Discontinous Percoll gradients consisting of 5 distinct bands with densities of 1.0, 1.03, 1.05, 1.07 and 1.09 g/ml respectively were prepared by mixing 90% Percoll and M199H in differing proportions as described in Table 2.1. Gradients were formed by layering the bands, either by hand or using the meniscus follower (Fig 2.1), in which case only the left hand chamber of the gradient former was used. Bands were layered, commencing with the 1.09 g/ml layer. Gradients were stored, usually overnight, at 4°C until used.

b) Calibration of preformed gradients:

Discontinous and continuous Percoll gradients were calibrated using cross-linked dextran beads with accurately predetermined densities(Density Marker Beads, Pharmacia, U.K.), covering a range from 1.017 to 1.142 grams/ml. Gradients were centrifuged under the same conditions as for cell purification and the positions of the bands noted. The results(Fig 2.2) showed that whilst the continuous gradients gave a wider range of separation, with the bands usually clearly separated from each other, the discontinous gradients gave much clearer and sharper bands. The discontinous gradients also provided much sharper definition of the different bands of cells found during purification of Leydig cells, and therefore these gradients were chosen for the majority of cell preparations.

c) Leydig cell purification with Percoll gradients:

Unpurified or "crude" interstitial cells were prepared(see section 2.2.1 above) and approximately 120 x 10^6 cells layered onto each Percoll gradient. Gradients were then centrifuged at 4°C for 20 min at 800 x g in a Mistral 2L centrifuge and the Leydig cells, which focus in the lower of the three cell bands, were recovered by

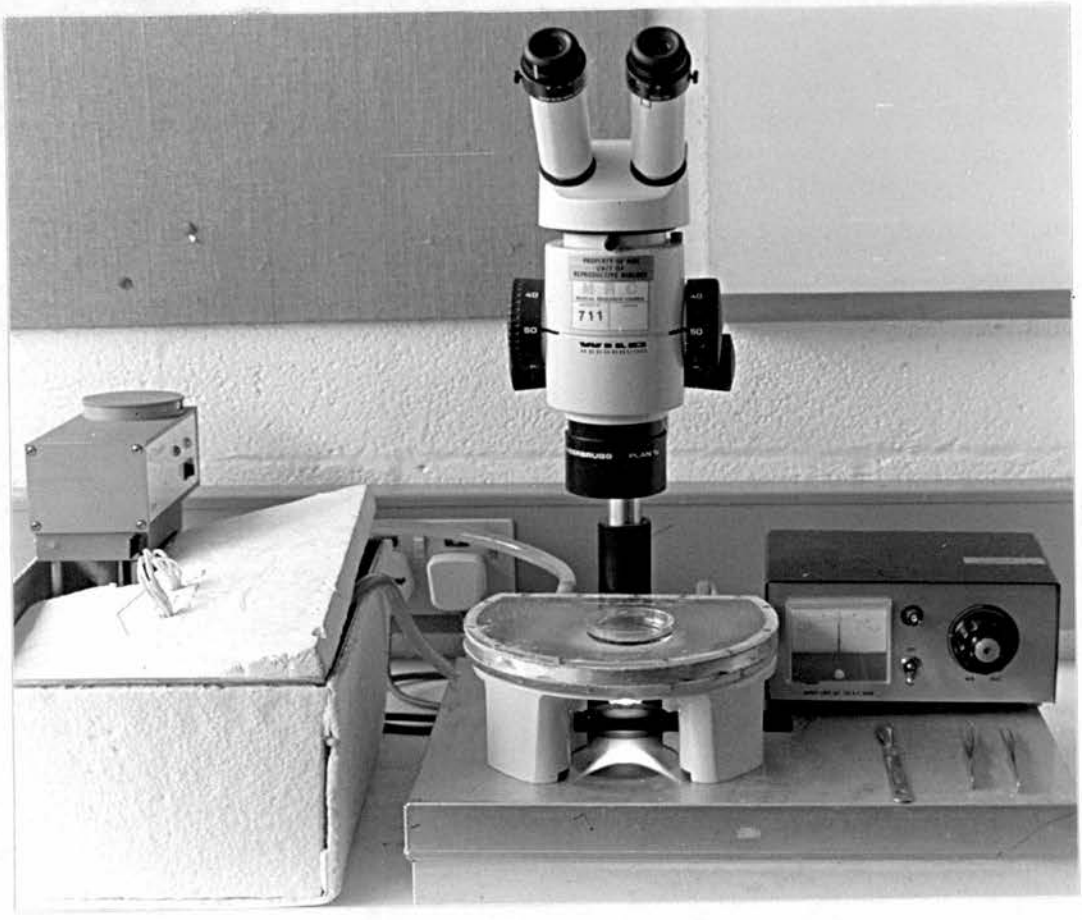
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Fig 2.3: Apparatus for the dissection of seminiferous tubules: The insulated water bath on the left, was filled with a mixture of ice and water which was continously pumped through the perspex stage throughout the dissection procedures. The microscope was adjusted for trans-illumination of the sample being dissected.

aspiration. Recovered cells were washed in M199H and harvested by centrifugation at 4°C for 5 minutes at 250 x g. The cell pellets, containing 70-90% Leydig cells, were then resuspended in M199H and counted using a Neuberg Haemocytometer. Cells were then stored at 4°C until used in the procedures described below.

2.3: Preparation of seminiferous tubules:

2.3.1: Collagenase dispersion of testes:

Groups of 4 testes were decapsulated and dispersed for 3-4 min using 1.5mls/testis of medium 199(Flow Laboratories) containing Hank's salts and 20mM Hepes buffer(M199H pH 7.4), 0.25mg/ml collagenase(Sigma type I) and 2.5mg/ml bovine serum albumin(BSA, Sigma, Fraction V) in a shaking water bath(170 cycles/minute) at 32°C. After dispersion the tissue was diluted with 50 mls M199H and the tubular mass allowed to settle under gravity. The supernatant was decanted and either discarded or used for Leydig cell preparation(see above). The seminiferous tubules were washed with a further 50 mls of M199H and stored on ice to provide a pool of material for dissection.

2.3.2: Dissection of seminiferous tubules:

All stages of the dissection procedure described below were performed on a trans-illuminated 1/2 inch deep perspex stage, mounted on a Leitz dissecting microscope, and through which ice cold water was pumped continuously(Fig 2.3). Seminiferous tubules were either collagenase dispersed before dissection(see above) for use in static cultures or perifusions, or in the case of tubules dissected for testosterone or protein determination, lengths were teased out from decapsulated undispersed testes.

In the latter case whole decapsulated testes were placed in plastic Petri dishes with about 5-10 mls M199H, manually teased

apart using watchmakers forceps, and 2-10 cm lengths of tubules isolated. Collagenase dispersed testes were processed similarly. Tubules with adherent interstitial/connective tissue were not used. The 2-10 cm lengths of seminiferous tubules were pooled in a Petri dish containing 2-5 mls ice cold M199H. When a sufficient pool of tubules had been obtained, all but a thin film of the medium was aspirated and the dish placed above a transparent 1 mm squared grid, on the microscope stage. Tubules were aligned above this grid in parallel using watchmakers forceps and cut into 0.5 cm lengths using a scalpel. These 0.5 cm sections were then used for either co-incubation with Leydig cells, preparation of conditioned medium, co-perifusion with Leydig cells or for determination of their protein and testosterone content(see below).

2.3.3: Stage dissection of seminiferous tubules:

Using transillumination microscopy the spermatogenic cycle as described by Leblond & Clermont(1952b) can be readily dissected into four major pools according to the appearance of the tubule(Parvinen & Vanha-Pertulla, 1972). The stages of the spermatogenic cycle were thus separated into four groups:- VII-VIII, which appear dark in the centre of the tubule, stages IX-XII, which appear pale, stages XIII-I which appear weakly spotted and stages II-VI which appear strongly spotted under transillumination microscopy. Using identical techniques to those described above(section 2.3.2), seminiferous tubules were dissected according to the stage of the spermatogenic cycle(stage dissected tubules), and used for the procedures described below. Due to the varying lengths of these stages it was not possible to consistently dissect out 0.5 cm lengths of each stage but the total lengths of tubule used were kept constant in any particular

experiment.

2.3.4: hCG binding to dissected seminiferous tubules:

a) hCG binding assay:

To either 10 cms of seminiferous tubules(20 x 0.5 cm lengths) or 50,000 Percoll-purified Leydig cells in 200 µl M199H was added either 50 µl of unlabelled hCG(100 IU/ml; Chorulon) in M199H to determine non-specific binding or 50 µl of M199H alone, and to all tubes 50 µl of I-125-hCG(~100,000 c.p.m; iodinated-hCG CR119; NIAMDDK, U.S.A., gifted by Dr. T.A. Bramley) was added. Tubes were incubated at room temperature for 18 h, then diluted with 3 mls 0.9%(w/v) saline and centrifuged at 4°C for 5 min at 800 x g. Supernatants were decanted and precipitates counted in an NE1600(New England Nuclear, U.S.A) gamma-counter. Specific binding of hCG was expressed as a percentage of total counts.

2.3.5: Diffusion of testosterone from isolated seminiferous tubules:

10 cm aliquots(20 x 0.5 cm) of seminiferous tubules were placed in 2 mls of M199H at 20°C. At various times thereafter the tubules were removed from the medium and both tubules and medium were extracted and assayed for testosterone content.

2.4: Co-incubation of seminiferous tubules and Leydig cells:

For tissue co-incubation, varying lengths(1,2,4,8 & 10 cm) of dissected seminiferous tubules(see section 2.3 above) were transferred in 0.5 cm segments to 24-well plastic culture dishes(Nunc, Denmark) to each well of which was added 200 µl medium 199 containing Earles' salts(Flow Laboratories, U.K.) 2 mM L-glutamine(Flow Laboratories, U.K.), transferrin (5 µg/ml; Sigma), insulin(10 µg/ml; Sigma), ceruloplasmin(1 I.U./ml; Sigma), penicillin(100 I.U./ml, Flow Laboratories U.K.), streptomycin(100 µg/ml; Flow Laboratories U.K.),

fungizone(2.5µg/ml; Flow Laboratories U.K.) and BSA(=M199E). Concentrated suspensions of either unpurified interstitial cells or Percoll-purified Leydig cells(see section 2.2 above) were diluted and aliquots of 0.05-0.5 x 10^6cells/well were added in 250 µl M199E. Human chorionic gonadotrophin(hCG, Chorulon, Intervet) was added to a final concentration of 0, 0.2 or 2,000 mI.U./ml and the plates incubated in a total volume of 0.5 mls for various periods of time at 32°C under a humidified atmosphere of 5%CO_2:95% air. In some experiments muscle tissue was also incubated with cells and in all experiments cells, incubated with medium alone were used as a control. For some experiments, parallel incubations were prepared and incubated for 24 h in the absence of hCG and the medium then aspirated and replaced with fresh M199E containing hCG prior to a further 5 h incubation. At the end of the incubations all media were aspirated, centrifuged at 1,000 x g for 5 min at 4°C to remove cellular debris, and then stored at -20°C prior to measurement of testosterone by radioimmunoassay.

2.5: Seminiferous tubule-conditioned medium:

2.5.1: Preparation of seminiferous tubule-conditioned medium:

Tubules were dissected as described above and 20 x 0.5 cm lengths/ml(i.e. 10 cm/ml) were transferred into M199E in 5 ml aliquots and incubated for 16-24 h at 32°C under a humidified atmosphere of 5%CO_2:95% air. In some experiments seminiferous tubules were prepared as described above and transferred into medium to give final levels of either 5 cm/ml(10 x 0.5 cm lengths), 10 cm/ml(20 x 0.5 cm lengths), 20 cm/ml(40 x 0.5 cm lengths) or 40 cm/ml(80 x 0.5 cm lengths). After incubation the medium was decanted and centrifuged(1,000 x g for 5 min at 4°C) to remove the seminiferous tubules. The supernatant was

then mixed with 2 mg/ml activated charcoal(Norit A, Sigma) for 30-60 min at 4°C to remove steroids, and then centrifuged at 4°C for 30 min at 1,000 x g. The supernatant was decanted and stored at -20°C until used for the procedures described below. Aliquots of medium alone were incubated and processed as described above and were used as a control. As an additional control, strips of thigh muscle were also incubated and this muscle-conditioned medium used in the experiments described below.

2.5.2: Effect of tubule-conditioned medium on Leydig cells:

Seminiferous tubule-conditioned medium, muscle-conditioned medium and control medium were tested for their ability to alter basal and hCG-stimulated testosterone production by Percoll-purified Leydig cells in vitro. Conditions were identical to those described above for tissue co-incubations except that instead of tubules being added directly, a final volume of 50% of the test medium was added to each well. Cells were incubated in the presence of this test medium, as described above, for either 5 h in the presence of hCG or for 24 h without hCG, following which the medium was changed and replaced with medium containing hCG and incubation continued for a further 5 h. At the end of each experiment samples were stored at -20°C prior to measurement of testosterone by radioimmunoassay.

2.6: Concentration of seminiferous tubule-conditioned medium:

In an attempt to concentrate any factor(s) present in seminiferous tubule-conditioned medium two experiments were performed in which medium was concentrated by stepwise ammonium sulphate precipitation. All medium was charcoal-extracted prior to treatment. An equal volume of medium alone was also precipitated as a control.

In the first experiment, a volume of 20 mls of control and

seminiferous tubule-conditioned medium were precipitated stepwise with increasing concentrations of ammonium sulphate(pH6.5, BDH).

Step 1: To each 20 ml aliquot of medium were added 13.4 mls of saturated ammonium sulphate, bringing the solution to a final concentration of 40% ammonium sulphate. These solutions were stirred for 2 h at 4°C prior to centrifugation at 45,000 x g for 30 min at 4°C in an ultracentrifuge(Sorval, U.K. Ltd). The pellet was resuspended in 2 mls M199H and the supernatant taken on to step 2.

Step 2: To each 33.4 ml supernatant from step 1 were added 16.6 mls of saturated ammonium sulphate, raising the concentration of ammonium sulphate to 60% . These solutions were stirred for 2 h at 4°C prior to centrifugation as for step 1. The pellets were resuspended in 2 mls M199H and the supernatant taken on to step 3.

Step 3: To each 50 ml supernatant from step 2 a further 50 mls of saturated ammonium sulphate was added, bringing the final ammonium sulphate concentration to 80%. These solutions were stirred overnight at 4°C prior to centrifugation as for steps 1 and 2. The supernatant was then discarded and the pellets resuspended in 2 mls M199H.

All precipitated fractions and control media were then desalted using Sephadex PD-10 columns(Pharmacia, U.K. Ltd), and dialyzed for 12 h at 4°C against M199H prior to storage at -20°C until used in the incubation system described above(see section 2.5.2).

In a second experiment a volume of 100 mls of control and seminiferous tubule conditioned medium were precipitated stepwise with increasing concentrations of ammonium sulphate adjusted to pH 7.4. Essentially, the precipitation was as described for the first experiment, except that after the pellets were resuspended in 2 mls M199H they were dialysed against M199E for 40 h at 4°C,

then made up to 5 mls in M199E and frozen prior to use in the incubation system described above.

2.7: Co-perifusion of seminiferous tubules and Leydig cells:

2.7.1: Preparation of Leydig cells and seminiferous tubules:

Seminiferous tubules were prepared as described above, between 50-400cm of tubules being required for each column. The optimum number of seminiferous tubules was found to be 200 cm per column and the methods described therefore make use of this observation. For each column, between 1.5 and 3 million Percoll-purified Leydig cells were used, and these were prepared as described above.

2.7.2: Preparation of materials for perifusion:

For each column 0.5 g of Bio-Gel P-2(200-400 mesh, Bio-Rad Laboratories, U.S.A.) was preswollen for 18 h in physiological saline (0.9% w/v sodium chloride, Steripak Ltd, U.K.). Medium 199 containing Earles' salts(Pharmacia Ltd U.K.) was gassed for 20-30 min with 95% O_2:5% CO_2(British Oxygen Ltd) prior to the addition of 1 mg/ml BSA, 2 mg/ml glucose(D-glucose, BDH Chemicals Ltd, U.K.), 0.1 mg/ml L-glutamine(BDH Chemicals Ltd, U.K.) and 20 µg/ml Garamycin(gentamicin sulphate, Schering Corporation, U.S.A). The pH of the medium was adjusted to pH 7.4 using 0.1M sodium hydroxide. oLH(NIH-LH 23; 2.3 SI units/mg) was diluted in medium to give doses ranging from 0.01-10 ng/ml as required. Columns were prepared by inserting a 0.8 x 40 mm needle(Becton Dickinson Ltd, U.K.) through the plunger of a 2 ml plastic syringe(Gillette Surgical, U.K.) and sealing the syringe with nylon guaze. (Lowry & McMartin, 1974)

2.7.3: Perifusion:

For each column 1.5-3 million Leydig cells were mixed with the preswollen Bio-Gel. To some columns at this stage 50-400 cm of 0.5 cm

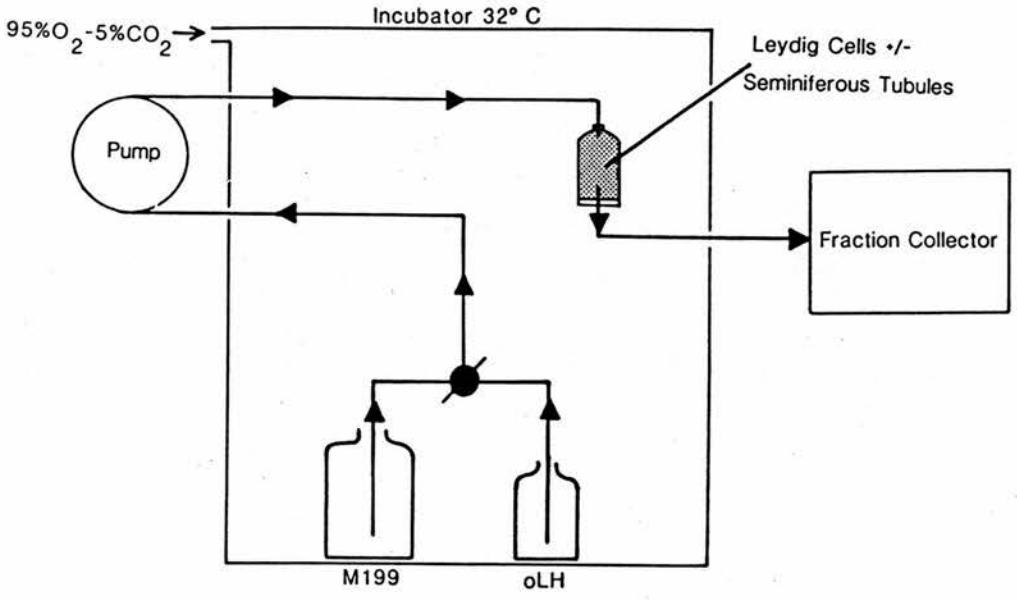


Fig 2.4: Apparatus for Perifusion experiments: Medium 199 containing oLH(oLH) or medium alone(M199) were stored in the incubator along with columns containing either Leydig cells or Leydig cells with seminiferous tubules, or seminferous tubules alone. The multi-channel pump perifused the medium thorough the columns and thence into the fraction collector shown at the right. The flow of either M199 or LH into the columns was controlled by a three-way tap.

lengths of seminiferous tubules were added. The Bio-gel suspensions were loaded into the syringes by suction and the contents of the columns allowed to settle while medium prepared as described above was perifused through the system by a multichannel pump(Ismatec mp-25, Ismatec SA, Switzerland; Fig 2.4). After loading, columns were allowed to settle for 30min before perifusate samples were collected every 10 min using a multichannel fraction collector(LKB UltroRac II, LKB, Sweden). By varying the setting on the pump the perifusion speed was varied between experiments from 0.25-0.5 mls/min. In most experiments columns were perifused for 2 h without LH stimulation and then 'pulsed' with LH for 10 min at 2 and 4 h after commencement of the perifusion. Experiments were terminated after 6 h as previous experiments have shown that crude interstitial cell preparations begin to deteriorate after this time(Wu, Zhang, Williams & deKretser, 1985). Samples were stored at -20^{o}C prior to measurement of testosterone by radioimmunoassay.

2.8: Collection of body fluids and samples for testosterone determination:

2.8.1: Interstitial fluid:

Collection of interstitial fluid from the testis was performed using techniques described by Sharpe & Cooper(1983). The testes were isolated after death and a small incision was made in the testicular capsule at the caudal end of the testis(the point furthest from the rete testis). The testis was then placed in a plastic centrifuge tube at 4^{o}C and interstitial fluid allowed to drain from the testis over 16 h under gravity. After collection of fluid the testis was removed and used for determination of testis testosterone content, whilst the interstitial fluid was centrifuged at 1,000 x g, 4^{o}C for 15 min to

remove any contaminating red blood cells. Interstitial fluid volume was determined by aspiration of the interstitial fluid in 10 µl aliquots.

2.8.2: Serum:

Serum was obtained at death by collecting trunk blood in non-heparinised collection tubes. After storing at 4°C for 12-16 h and centrifugation at 1,000 x g for 10 min at 4°C in a Mistral 2L centrifuge, serum was frozen at -20°C prior to measurement of LH, FSH, and testosterone.

2.8.3: Whole testes:

Whole testes were either decapsulated immediately after death or after collection of interstitial fluid and tissue was stored at -20°C until extracted for testosterone determination. Testicular weight was determined by preweighing the tubes in which the testes were collected, then re-weighing them after collection of the testes.

2.8.4: Seminiferous tubules:

Seminiferous tubules were dissected as described above(see sections 2.3.3-4 above) and 10 or 20 cm aliquots placed in 0.3 mls distilled water to cause lysis of the tissue. Samples were then stored at -20°C, and were subsequently thawed and sonicated for 60 sec using an MSE Soniprep 150(MSE Instruments Ltd, U.K.), prior to protein estimation or extraction for testosterone assay.

2.9: Testosterone radioimmunoassays:

For the purposes of these studies a novel iodine-based radio-immunoassay was validated. The assay previously in use in these laboratories was a tritium-based radioimmunoassay previously validated by Corker and Davidson(1978). This assay was used for comparison with the new radioimmunoassay. Antiserum for the latter assay was kindly

donated by Dr. Brian Morris(ABRO) and the testosterone 3-carboxy-methyl oxime for iodination was donated by Gerry Norblom(U.S.A).

2.9.1: Tritium radioimmunoassay:

The assay was performed using 0.075M phosphate-buffered saline with 5% gelatin added(Type I, Sigma U.K., pH 7.4, PBGS). The antiserum for this assay was a gift from Dr. S. Tilson and was raised in the goat against testosterone 3-carboxy-methyl oxime-BSA. Assays were performed in a final volume of 0.3 mls PBGS buffer, comprising 100 µl of sample, 100 µl of a 1:6,000 dilution of the antiserum, and 100 µl of 1,2,6,7-^{3}H testosterone(Amersham International Ltd, U.K.) containing approximately 8,000 counts per minute. Samples were incubated either at room temperature for 1 h or overnight at 4^{o}C. After incubation samples were separated on ice using dextran-coated charcoal(DCC) in PBGS(2.5 mg dextran sulphate, Pharmacia, U.K., 250 mg activated charcoal, Norit A, Sigma Ltd. U.K./100 mls PGBS). 1 ml of DCC was added to each tube, the tubes vortexed and allowed to stand on ice for 15 min. Bound and free hormone were then separated by centrifugation at 1,500 x g for 10 min at 4^{o}C. The supernatant was decanted into plastic scintillation vials containing 10 mls Triton: toluene scintillant(10 g PPO, 0.75 g POPOP in 2.5 l toluene and 1.25 l Triton X100), prior to counting in a β-counter. Results were then calculated using a computer programme written for a desk top Hewlett-Packard computer. The programme was provided by Dr. Sharpe.

2.9.2: Iodination of tracer for the iodine-testosterone assay:

2 µg testosterone-3-carboxymethyl oxime-histamine was resuspended in 40 µl 0.5 M phosphate-buffered saline(PBS, pH 7.5) prior to addition of 2 millicuries Na-I-125(Amersham International Ltd, U.K.). Iodination was initiated by the addition of 10 µl of Chloramine-T

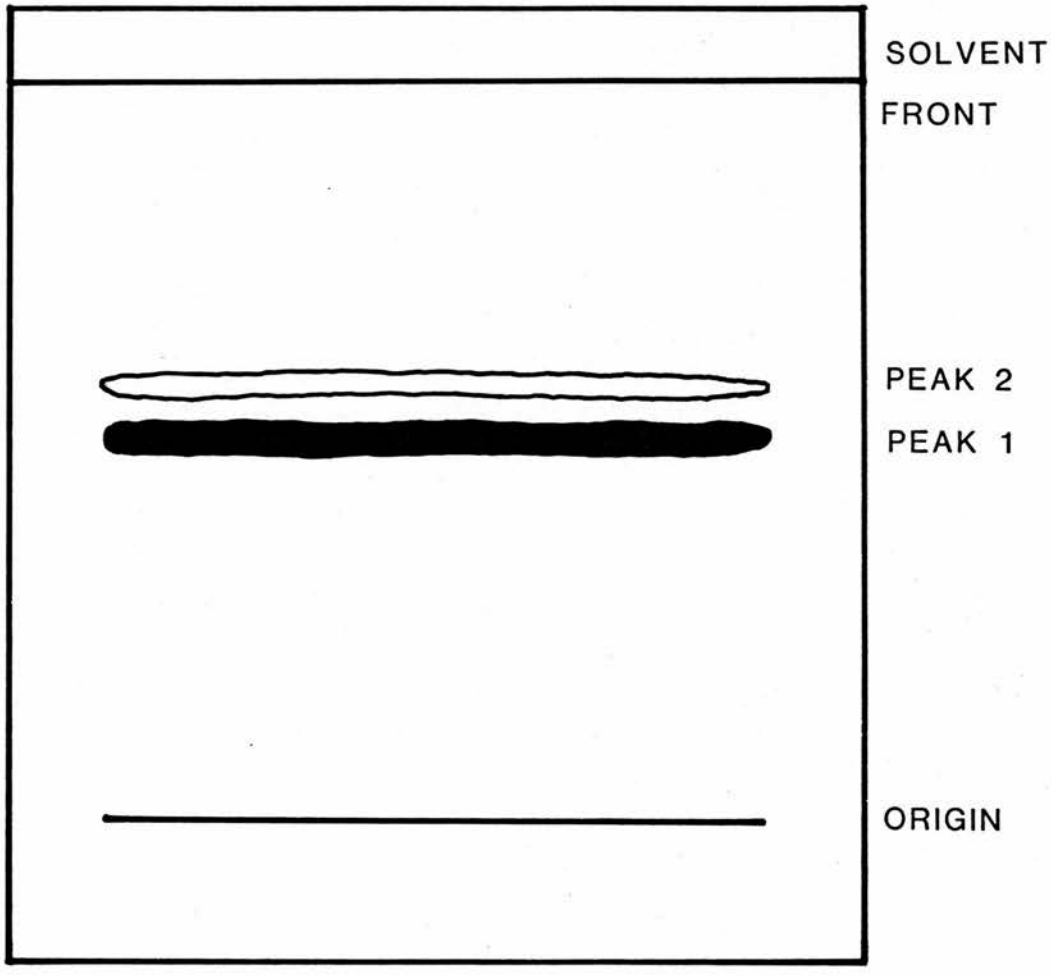


Fig 2.5: Thin Layer Chromatography of iodinated testosterone-3-CMO conjugate for the iodine testosterone radioimmunoassay. The peak containing the most active product is shaded(Peak 1).

(1 mg/ml BDH Chemicals Ltd, U.K.) in 0.05M PBS, and the reaction allowed to proceed for 2 min before the addition of 10 µl sodium meta-bisulphite(1 mg/ml, BDH Chemicals Ltd, U.K.) in 0.05M PBS. Subsequent to this reaction a further 0.5 mls of 0.05M PBS were added to 'bulk up' the solution. The solution was then extracted twice with 0.5 mls ethyl acetate(BDH Chemicals Ltd, U.K.), by vortexing for 60 sec. The ethyl acetate layer was allowed to separate and was then pipetted into another vessel. The organic phase was then dried down to about 50-100 µl and spotted onto a TLC plate(Kieselgel 60F254, Merck, West Germany). Products were then separated by thin layer chromatography using a mixture of Chloroform:Methanol:glacial acetic acid(90:10:1). The plate was run until the solvent front was within 2 cm of the top of the plate. The required material was detected using a handheld Geiger-counter with a 1 mm window, and was eluted from the TLC plate overnight in ethanol and stored at 4^{o}C until used. The average yield from 10 iodinations was 33+/-12%. After TLC, 2 bands of iodinated material were detectable(Fig 2.5) and the lower of these bands contained the more active material which was used for the radio-immunoassay.

2.9.3: Assay method for the iodine-testosterone assay:

For assay, 0.1 ml sample was added to 0.5 ml PBGS followed by 0.1 ml of a 1:350,000 dilution of the testosterone antiserum and approximately 10,000 c.p.m. of I-125 labelled testosterone-3-CMO. In each assay testosterone standards were included ranging from 5-640 pg/tube, and high and low quality controls were included to assess the reproducibility of the assay. Tubes were incubated at room temperature for 2 h before the addition of 0.1 ml of a 1:1,000 dilution of normal sheep serum and 0.1 ml of a 1:25 dilution of goat anti-sheep

precipitating serum to precipitate immunoglobulins. Both the normal sheep serum and the second antibody were provided by the Scottish Antibody Production Unit. Following addition of the second antibody tubes were incubated overnight at 4°C and then centrifuged for 30 min at 1,500 x g, the supernatant decanted and the precipitate counted in a gamma counter(NE 1600, Nuclear Enterprises Ltd, U.K.), linked to a Commodore 4032 micro-computer(CBM computers Ltd, U.K.). Results were calculated using this system.

2.9.4: Validation of the iodine-testosterone assay:

i) Double dilution of antiserum:

The recommended dilution of the antiserum for the assay was 1:350,000, but to test the optimal dilution the antiserum was double-diluted throughout the range 1:22,000 - 1:2,867,200,000. The results (not shown) suggested that the antiserum dilution recommended was far from optimal, and also that if necessary the sensitivity of the assay could be markedly improved. However, for the purpose of the present studies, the sensitivity of the assay, covering a range of 5-640 pg/tube was judged to be satisfactory and therefore the recommended antiserum dilution was adopted for routine assay procedures.

ii) Parallel dilution curves:

Double dilution of testosterone standards and rat testicular interstitial fluid and whole testis extracts produced curves parallel to each other, as judged by 2-factor analysis of variance with replication(Data not shown).

iii) Extraction and chromatography of assay quality controls:

To provide a constant measure of the reliability of the assay a quality control was included in each assay. The quality control for this assay was prepared from a whole testis incubate

containing >1,000 ng/ml testosterone. To assess both the purity of this quality control and to compare the iodine assay with the tritium assay which was then in use these quality controls were extracted and separated using the following procedures.

a) Hexane:Ether extraction:

0.5 mls of the quality control was mixed with 6.5 mls hexane:diethyl ether(4:1 v/v) and the aqueous phase frozen in a dry ice:ethanol bath. The organic phase was decanted and both the organic and aqueous phases dried down and resuspended in 1 ml PGBS. Samples from the unextracted, aqueous and organic phases were then assayed in both the tritium and iodine radioimmunoassays.

b) Column separation:

0.2 mls of the quality control was mixed with 2.5 mls hexane:diethyl ether(4:1 v/v) and the aqueous phase frozen, decanted and dried down as described above. The organic phase was resuspended in 0.5 mls iso-octane and left overnight. Celite 545(Johns Manville Co. U.S.A) was activated overnight at 600°C and packed into glass columns. The sample was loaded onto the column in 0.5 mls iso-octane and then fractions eluted as follows:

Fraction 1) Eluted with 3.5 mls iso-octane:
Androstenedione fraction.

2) Eluted with 3.5 mls 5% benzene in iso-octane:
Dihydrotestosterone fraction.

3) Eluted with 3.5 mls 10% benzene in iso-octane: Waste

4) Eluted with 3.5 mls 20% benzene in iso-octane:
Testosterone fraction.

5) Columns washed with 5 mls ethanol: Waste.

All column fractions were dried down and resuspended in 1 ml PGBS

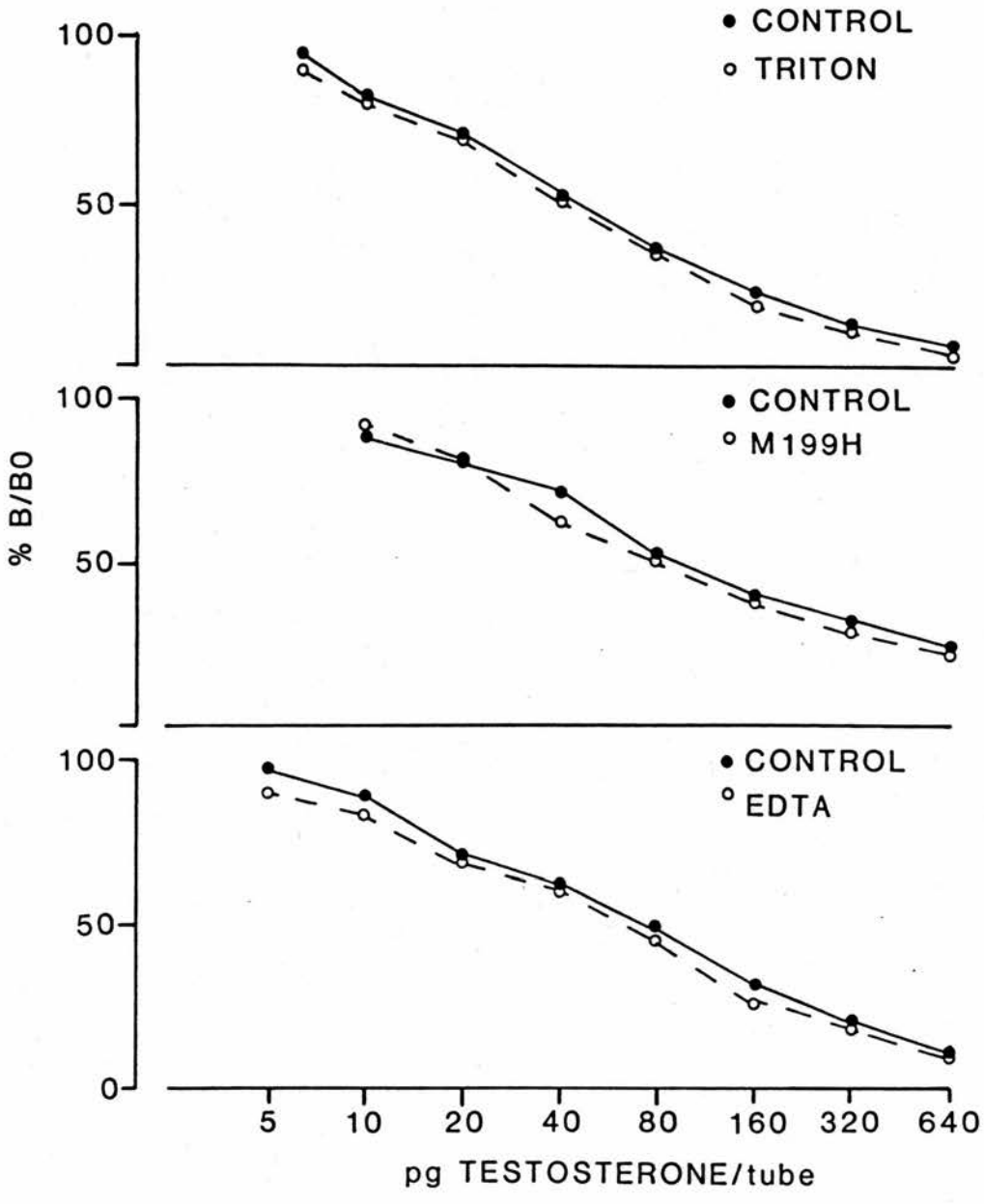


Fig 2.6: The effect on the standard curve of adding 0.2% Triton(Top panel), 10% M199H(Centre panel) or EDTA(Bottom panel) to the assay buffer for the iodine testosterone radioimmunoassay. None of the treatments displaced the standard curve.

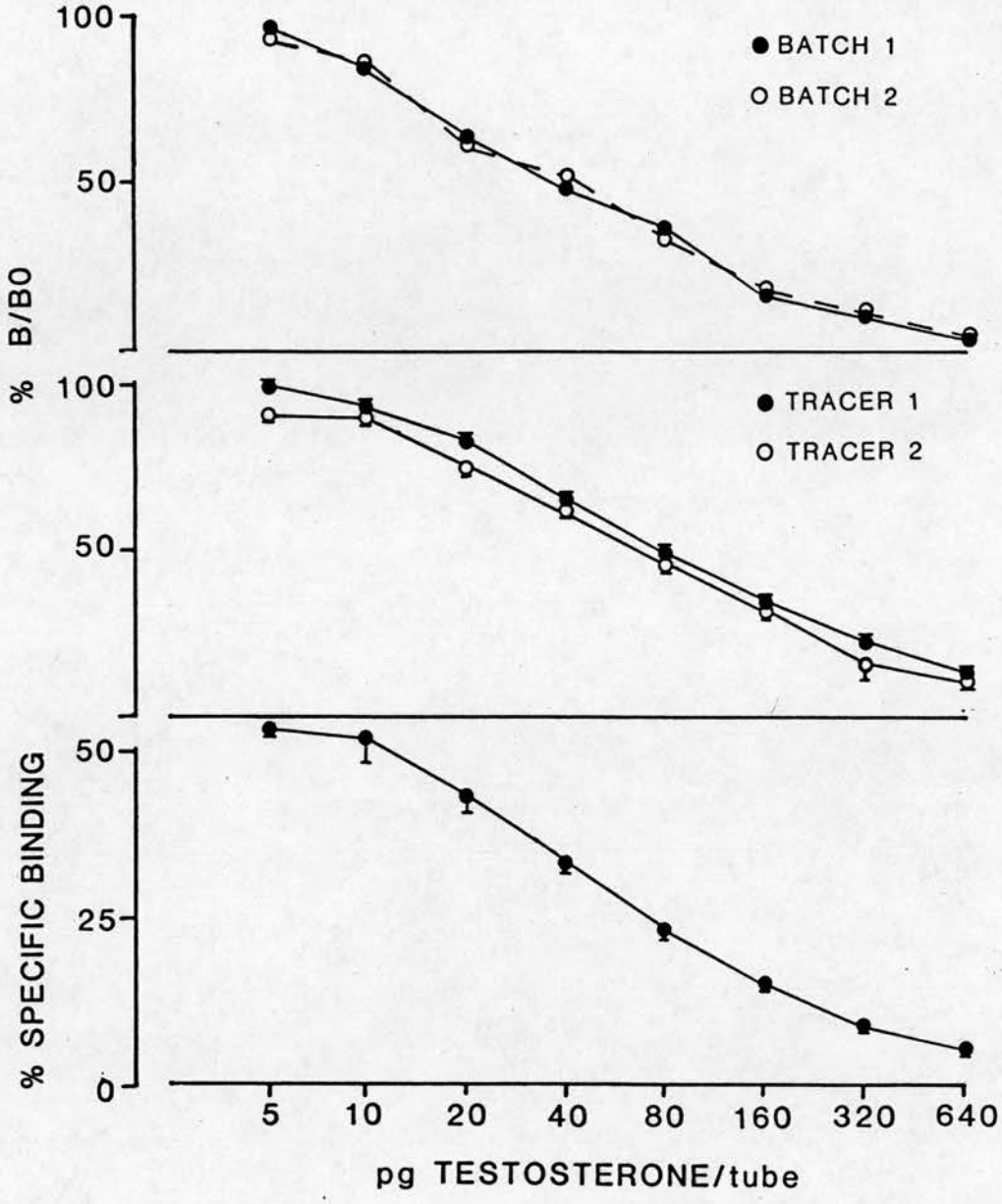


Fig 2.7: Effects of changing the batch of antiserum(Top panel) or tracer(Centre panel), on the standard curve for the iodine testosterone radioimmunoassay. The reproducibility of the standard curve is indicated in the bottom panel, which shows the composite curve(mean+/-s.d.) from 7 successive assays.

and assayed in both tritium and iodine radioimmunoassays along with unextracted controls. Results from both assays agreed closely, both as to the total content and also as to the extracted content of testosterone in the quality controls(data not shown).

iv) Effects of medium, EDTA, and Triton on standard curves:

To reduce the background non-specific binding in the iodine assay, 0.2% Triton was added to the saline wash. The effects of this addition as well as the effect of 100 µl of M199H or EDTA were also assessed. Results(Fig 2.6) showed no alteration of binding with any of these treatments, although the addition of 0.2% Triton X-100 to the assay saline wash significantly reduced the amount of non-specific binding in the assay.

v) Consistency of the assay:

a) Comparison of 7 different standard curves:

To assess the reproducibility of the assay during a number of successive centrifugations, which would allow large numbers of samples to be assayed in one assay, seven different standard curves were averaged to determine the variation between centrifugations. Comparing seven standard curves over a 5 h period less than 6% variation between curves was seen overall(Fig 2.7).

b) Assessment of assay drift:

Comparing both high and low quality controls, along with zero binding tubes placed at the beginning and the end of seven different centrifugations, no difference was seen between zero binding at the beginning or end of the centrifugations(59.44% vs 59.41%). Neither was any difference seen in the percentage binding for the high(20.32% vs 20.40%) or low(50.64% vs 50.16%) quality controls.

STEROID	% CROSS-REACTION	SOURCE
Adrenosterone	<0.001	Sigma Ltd, U.K.
Aldosterone	<0.005	Sigma Ltd, U.K.
Androstenedione	1.6	Calbiochem
Cholesterol	<0.001	Steraloids, U.K.
Cortisol	<0.001	Steraloids, U.K.
Corticosterone	<0.005	Steraloids, U.K.
Deoxycortisol	<0.001	Steraloids, U.K.
Dehydroepiandrosterone	<0.001	Sigma Ltd, U.K.
5αDihydrotestosterone	23.0	Sigma Ltd, U.K.
5βDihydrotestosterone	<0.002	Sigma Ltd, U.K.
17α Hydroxyprogesterone	<0.001	Sigma Ltd, U.K.
Oestradiol 17α	<0.01	Sigma Ltd, U.K.
Oestradiol 17β	<0.01	Koch-Light & Co Ltd, U.K.
Estriol	<0.001	Calbiochem
Oestrone	3.1	Sigma Ltd, U.K.
Pregnenolone	<0.001	Sigma Ltd, U.K.
Progesterone	<0.001	Sigma Ltd, U.K.
17α testosterone	<0.02	Steraloids, U.K.
19 Hydroxytestosterone	0.17	MRC Steroid Collection.
7α Hydroxytestosterone	0.30	MRC Steroid Collection.
16α Hydroxytestosterone	0.48	MRC Steroid Collection.
Bovine Serum Albumin	0.000001	Sigma Ltd, U.K.
Cyproterone Acetate	0.002	Keymer Pharmaceuticals.

Table 2.2: Crossreaction of various steroids in the iodine-testosterone radioimmunoassay. The percentage crossreaction of each of 19 steroids and BSA with reference to testosterone(100%) are shown.

c) Comparison of different preparations of tracer:

Using two different preparations of I-125 testosterone the reproducibility of results between batches of tracer was tested. No difference between batches of tracer was evident(Fig 2.7).

d) Comparison of antiserum batches:

Using two different batches of #505 antiserum at the same dilution the variation in batches of antiserum was assessed. No difference in antiserum batches was seen(Fig 2.7).

vi) Cross-reactivities:

The antiserum used for the assay cross-reacted significantly with dihydrotestosterone (23.0%), oestrone(3.1%) and androstenedione(1.6%) but showed minimal(<0.1%) cross-reactivity with 19 other steroids & BSA, as shown in Table 2.2.

vii) Coefficent of variation and the limit of detection of the assay:

The inter- and intra- assay coefficients of variation were 10.4 and 6.5%, respectively, and the limit of detection(90% B/B0) was 11.0+/-2.9 pg(mean +/- standard deviation, n = 173).

viii) Comparison of iodine and tritium assays:

Comparison of testosterone values obtained for 342 medium samples run simultaneously in this assay and in a conventional 3-H-testosterone based assay(Corker and Davidson, 1978; Sharpe and Fraser, 1983) showed close overall correlation(r = +0.96) with no significant difference(p>0.1) between results obtained in the two assays.

2.9.5: Preparation of samples for iodine assay:

i) Culture medium:

Culture media from both static incubations and from

perifusion experiments were assayed directly as extracted and unextracted samples gave identical results(see above Section 2.9.4.ii).

ii) Interstitial fluid:

Interstitial fluid was diluted 1:20 in M199H and then assayed directly as extracted and unextracted samples gave identical results(see above Section 2.9.4.ii).

iii) Serum:

Serum testosterone was assayed after extraction with hexane-diethyl ether. Essentially 0.3 mls serum was mixed with 0.1 mls tritiated testosterone(approx 1,000 c.p.m, 1,2,6,7-3H-testosterone, Amersham U.K. Ltd)to monitor for recovery. The resulting mixture was extracted with 2 x 3 mls of hexane-diethyl ether(4:1 V:V), mixed, and the aqueous phase frozen in a dry ice-ethanol water bath prior to decanting the organic phase. The organic phase was then dried down and resuspended in PGBS.

iv) Seminiferous tubules:

Seminiferous tubules were prepared as described above (Section 2.8.4) and thereafter extracted using an identical procedure to that described for serum samples.

v) Whole testes:

Whole testes were extracted using the method described by de Jong, Hey and van der Molen(1974). Essentially, to a whole decapsulated testis 2 mls distilled water were added together with approximately 1,000 c.p.m. 3-H testosterone to assess recovery. Testes were then sonicated for 2 min and 5 mls of acetone added, mixed and the testes centrifuged at 1,500 x g for 5 min at 4^{o}C. The liquid phase was decanted and a further 2 mls of acetone added to the tissue

deposit, mixed and recentrifuged. The supernatant was added to the previous organic phase and the acetone evaporated under nitrogen. The remaining aqueous phase was extracted twice with 3 mls ether, the aqueous phase frozen in a dry ice:ethanol bath and the organic phase decanted. The ether extract was dried down and resuspended in 2 mls 70:30 v/v Methanol:water mixture. This solution was then partitioned twice with 2 mls of redistilled hexane. The methanolic layer was then dried down and resuspended in 0.4 mls PGBS buffer, prior to taking aliquots for assay and recovery.

2.10: Protein assay:

Protein assays were carried out as described by Lowry et al (1951). Essentially, 0.1 mls sonicated seminiferous tubules were made up to 0.6 mls with distilled water. Standards were prepared at 0, 20, 40, 80, 100, 150 and 200 µg/0.6 mls distilled water using BSA. To each sample 3 mls Folin's solution A(2% Na_2CO_3, 0.01% $CuSO_4$, 0.02% Sodium Potassium tartrate in 0.1 N NaOH) were added and the samples incubated at room temperature for 45 minutes. Subsequently, 0.3 mls freshly diluted Folin-Ciocalteu's Phenol reagent(1:2 v/v with distilled water) were added and samples mixed immediately on a vortex mixer. After a further 15 min at room temperature samples were read at 750 nm in a spectrophotometer and results calculated from the standard curve.

2.11: LH and FSH radioimmunoassay:

Serum levels of LH and FSH were measured by radioimmunoassay as described by Fraser & Sandow(1977), using radioimmunoassay kits supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases(NIAMDD, U.S.A.). I am grateful to Miss Irene Cooper for carrying out the LH & FSH assays required for this study.

2.12: Histology:

EDS-injected rats were killed at the following times after treatment: 6 and 12 h, on days 1 and 3, and at 1, 2, 3, 4, 6 and 10 weeks . Vehicle-treated control animals were killed at week 1. The testes of 2 rats from each of the above groups were fixed by vascular perfusion via the thoracic aorta using 5% glutaraldehyde, 3% formaldehyde and 0.01% trinitrophenol buffered in 0.1M cacodylate as described by Kerr, Mayberry and Irby(1984). All such perfusions were carried out by Dr. J.B. Kerr. The fixed testes were removed, trimmed of connective tissue and fat, decapsulated and, after measurement of their volumes by water displacement(Elias and Hyde, 1980), cut into cubes about 2mm square and fixed for 2-3 h in the same fixative. Following post-fixation for 2 h in 2% cacodylate-buffered osmium tetroxide, blocks were stained en bloc with uranyl acetate, dehydrated in graded ethanols and embedded in Epon-araldite. All blocks were cut at 1 μm using a Reichert OmU3 ultramicrotome and then stained with 1% Toluidine blue in 1% borax. Sections were examined and photographed using a Zeiss photomicroscope fitted with oil-immersion objectives. All sectioning, staining and photomicroscopy was carried out by Dr. J.B. Kerr and Mr. K. Donachie.

To assess the effects of EDS treatment upon spermatogenesis, transverse sections of seminiferous tubules were examined to detect the occurrence and frequency of abnormal patterns of germ cell maturation with particular attention to the stage of the spermatogenic cycle using the classification of Leblond and Clermont(1952b).

2.13: Statistics:

Statistical differences between treatments were assessed in the following manner:

For all static incubations(Chapters 3 & 4), results were analysed using a Students' t-test and significance values obtained from Students' t-distributions.

Perifusions(Chapter 5) were analysed by applying Students paired t-tests to the mean values from paired columns. For example, where 4 columns were run in a single experiment, 2 with Leydig cells alone and two containing both Leydig cells and seminiferous tubules, values for each 10 minute sample were meaned for each treatment group prior to statisical analysis. Significance values were obtained from the Students' t-distribution.

Results obtained for tubule testosterone and protein concentrations, serum LH, FSH and testosterone, testicular weight, interstitial fluid and whole testis testosterone were subjected to analysis of variance in the EDS experiments. Where changes in these parameters were assessed at different times after the administration of EDS(Chapter 7), the significance of differences from controls was assessed using Dunnets' t-test and the significance of differences between treatment times was assessed using Tukey's t-test. Results for animals treated with MEHP, MAA or anti-LH were determined using t-tests, and those for unilaterally cryptorchid animals using paired t-tests.

CHAPTER 3

SEMINIFEROUS TUBULE-LEYDIG CELL CO-CULTURES

3.1: Introduction:

The aim of these studies was to provide a system for the analysis of communication between the seminiferous tubules and Leydig cells. There is growing evidence pointing towards the importance of intratesticular factors in the control of spermatogenesis, with much of the recent data suggesting that modulation of Leydig cell steroidogenesis by factors secreted within the testis may be an important aspect of this control(see Ch 1). These local factors may act in conjunction with the anterior pituitary gonadotrophins to either modulate their actions or to alter Leydig cell responsiveness. The seminiferous tubules and the Leydig cells comprise the two major functional components of the testis and the dependence of spermatogenesis upon the testosterone produced by the Leydig cells is widely accepted(Russell & Clermont, 1977; Russell et al, 1981; Stevens and Steinberger, 1983). There are however no reliable systems for the detection and monitoring of factors communicating between these compartments. Using the methods described above(Chapter 2.1-4) a system for the co-culture of Leydig cells with dissected seminiferous tubules was assessed to determine its suitability for such studies.

3.2: Co-culture of isolated seminiferous tubules and Leydig cells:

Isolated seminiferous tubules at a concentration of 10(20 x 0.5) cm/well were co-cultured with Percoll-purified Leydig cells, whilst Leydig cells were cultured with dissected strips of thigh muscle as a control. Culture was for 5 h in the presence of hCG. Neither seminiferous tubules nor muscle caused any significant change in Leydig cell testosterone production(Table 3.1) when compared with Leydig cells alone.

	TESTOSTERONE PRODUCTION ($ng/10^6$ CELLS)	
	BASAL	hCG(2 I.U./ml) STIMULATED
TISSUE:		
Control	25+/-1	149+/-8
Muscle	31+/-3	154+/-14
S/T	20+/-2	131+/-17

Table 3.1: Effect of muscle tissue and seminiferous tubules(S/T) on Leydig cell testosterone secretion in co-culture. Control = Leydig cells only(Mean+/-s.d. of triplicate samples).

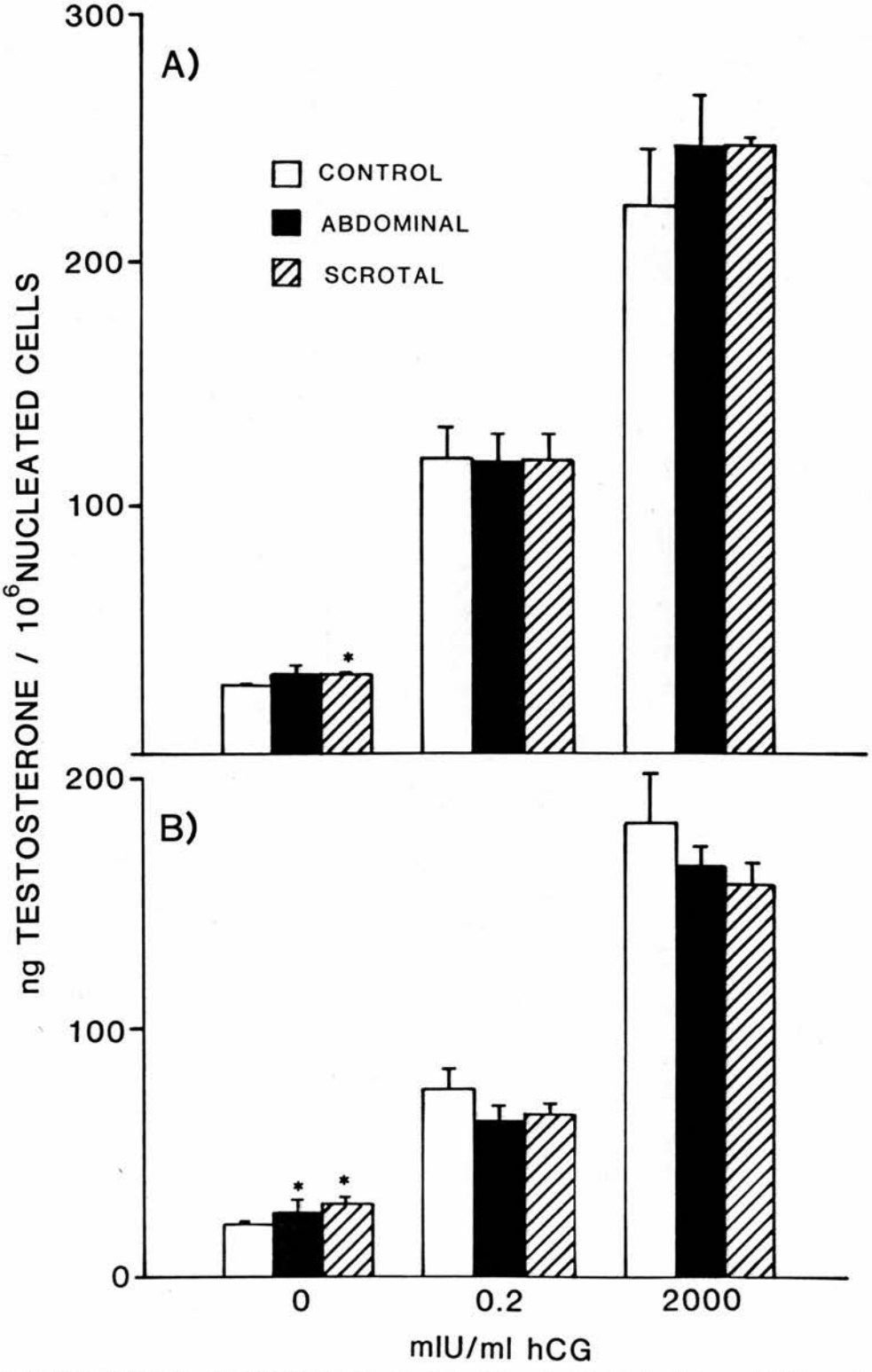


Fig 3.1: Effect of seminiferous tubules isolated from adult rats made unilaterally cryptorchid either 3(Panel A) or 5(Panel B) days earlier on Leydig cell testosterone secretion. * $p<0.05$ when compared with control(i.e. medium alone) values.
(Mean+/-s.d. of triplicate samples for both panels).

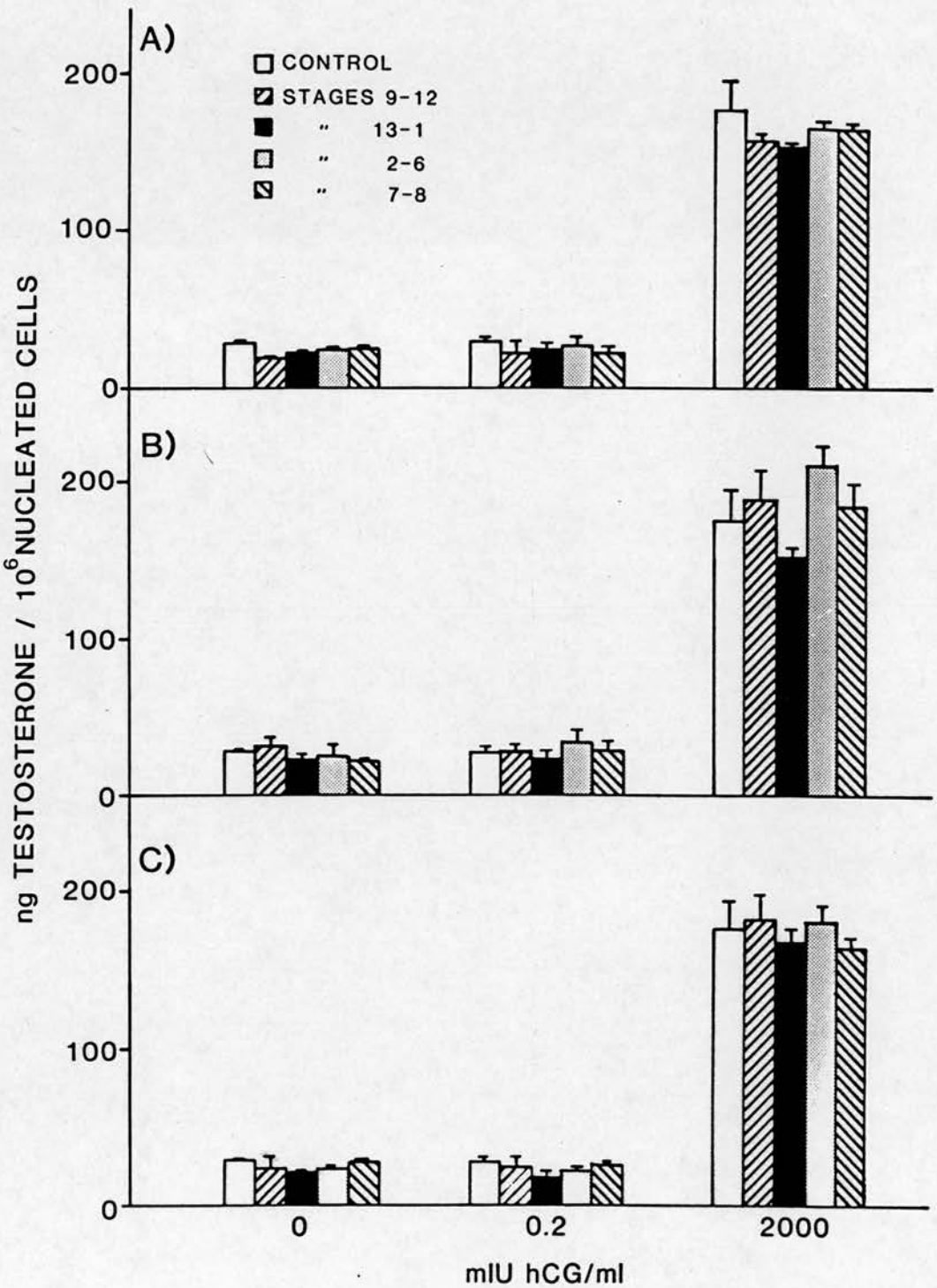


Fig 3.2: Effect of seminiferous tubules from normal rats, dissected by transilumination into different stages of the spermatogenic cycle, on Leydig cell testosterone secretion in vitro in the presence of hCG. Panel A: 1 cm seminiferous tubules/well. Panel B: 2 cm seminiferous tubules/well. Panel C: 4 cm seminiferous tubules/well. (Mean+/-s.d. of triplicate samples for all panels).

3.3: Co-culture of Leydig cells with isolated seminiferous tubules from unilaterally cryptorchid rats:

Co-culture of 4 cms(8 x 0.5 cm lengths) of seminiferous tubules dissected from either the abdominal(cryptorchid) or scrotal (normal) testes of adult rats, made unilaterally cryptorchid 3 or 5 days previously, with Percoll-purified Leydig cells isolated from age-matched untreated animals revealed no change in Leydig cell testosterone production after culture for 5 h in the presence of 0.2 or 2,000 mIU/ml hCG. However, seminiferous tubules from scrotal testes cultured with Percoll-purified Leydig cells in the absence of added hCG produced significantly($p<0.05$) higher levels of testosterone than did Leydig cells cultured in the absence of tubules for the three day post operation group(Fig 3.1). Seminiferous tubules from both scrotal and abdominal testes from rats at 5 days post operation when incubated with Percoll-purified Leydig cells enhanced Leydig cell testosterone production significantly($p<0.05$) in the absence of added hCG when compared with control Leydig cells(Fig 3.1).

3.4: Co-culture of Leydig cells with seminiferous tubules dissected according to the stage of the seminiferous epithelium:

Using the techniques for dissection of stages described above (Chapter 2.4) 1, 2 or 4 cms of stage dissected seminiferous tubules were co-cultured with Percoll purified Leydig cells, either without added hCG or in the presence of 0.2 or 2,000 mIU/ml hCG. No significant differences in Leydig cell testosterone production were observed in the presence of tubules from any of the grouped stages (IX-XII, XIII-I, II-VI or VII-VIII) at any dose of hCG stimulation, either with 4 cm(Fig 3.2.A), 2 cm(Fig 3.2.B) or 1 cm(Fig 3.2.C) of seminiferous tubules at each stage present in culture with the Leydig

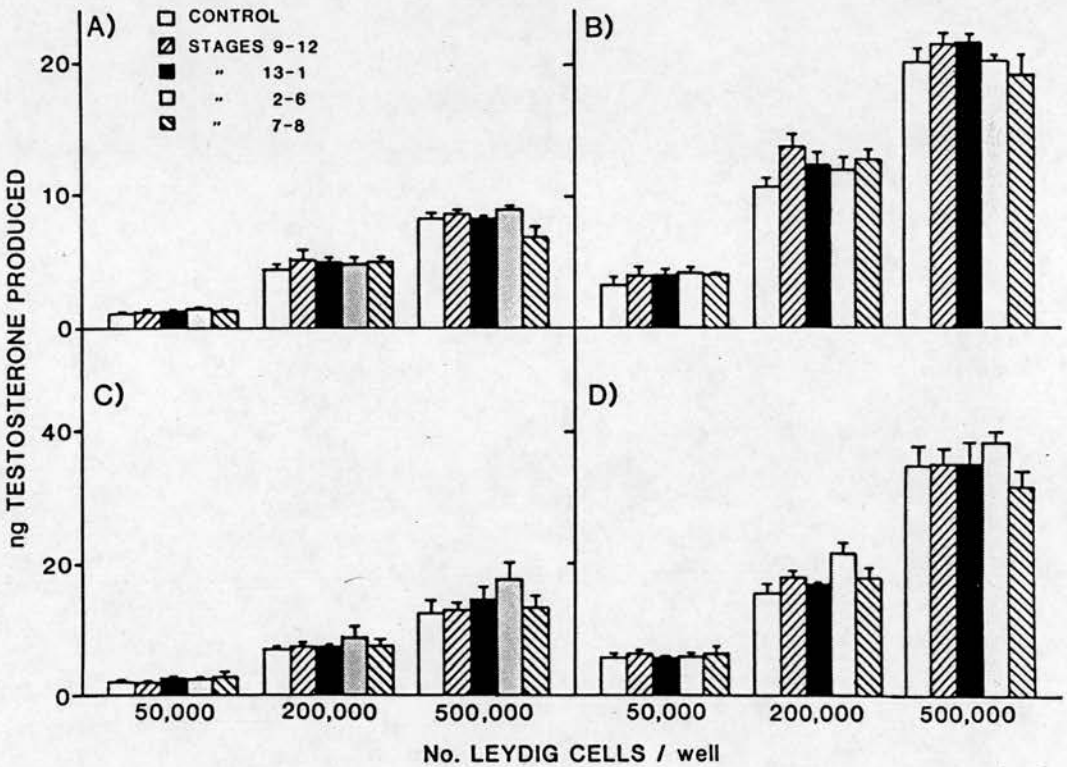


Fig 3.3: Effect of varying the ratio of Leydig cells:seminiferous tubules on basal Leydig cell testosterone secretion in the presence of seminiferous tubules dissected according to the stage of the spermatogenic cycle. Panel A: 1 cm seminiferous tubules/well, Leydig cell testosterone production over 5 h. Panel B: 1 cm seminiferous tubules/well, Leydig cell testosterone production over 24 h. Panel C: 2 cm seminiferous tubules/well, Leydig cell testosterone production over 5 h. Panel D: 2 cm seminiferous tubules/well, Leydig cell testosterone production over 24 h. (Mean+/-s.d. of triplicate samples for all panels).

cells.

To determine whether varying the time of culture or the ratio of seminiferous tubules to Leydig cells could improve the sensitivity of the method to any putative factors secreted by the seminiferous tubules, 1 or 2 cm of stage-dissected tubules were co-cultured for 4 or 24 h with 50,000, 200,000 or 500,000 Percoll purified Leydig cells/well in the absence of hCG stimulation. Despite varying the seminiferous tubule to Leydig cell ratio from 25,000 cells/cm tubules to 500,000 cells/cm tubules, no significant change in Leydig cell testosterone production was evident with any of the groups of stages after 4 h co-culture with either 1 or 2 cms(Fig 3.3.A & B) of seminiferous tubules per well. After 4 h the medium was changed and the co-culture continued for a further 20 h, and levels of testosterone produced over the whole 24 h period were calculated by summation of the results from 0-4 and 4-24 h. However, no significant change in Leydig cell testosterone production was induced by 1 or 2 cms of seminiferous tubules from any of the 4 stage groupings with 50-500,000 Leydig cells (Fig 3.3.C & D).

3.5: Discussion:

No effect of seminiferous tubules at any stage of the spermatogenic cycle on testosterone production by Leydig cells was evident during static co-cultures. Nor did muscle and Leydig cell co-cultures reveal any effects of muscle on testosterone production by Leydig cells during *in vitro* culture.

Due to the failure to demonstrate any significant reproducible effects of seminiferous tubules on Leydig cell testosterone production it must be concluded that either the seminiferous tubules do not affect Leydig cell testosterone production or the system used is not

suitable for the investigation of seminiferous tubule-Leydig cell interactions. However, in the light of reports from other laboratories (Parvinen et al, 1984). It would appear more probable that under the in vitro conditions tested the system is unable to reflect the subtle interactions between seminiferous tubules and Leydig cells in a consistent manner. Further modification of the culture conditions may be required for the establishment of a robust and readily reproducible system for the co-incubation of these tissues. Observations of a small effect of seminiferous tubules from cryptorchid animals on Leydig cell testosterone production(see 3.3 above) could be indicative of such communication, although no conclusions can be drawn from such variable effects.

If it is assumed that communication between seminiferous tubules and Leydig cells indeed exists, then the question arises as to why these cultures have failed to demonstrate such communication. Although tissue co-culture provides the most direct means for testing for tissue interactions there are a number of drawbacks inherent in the method. The primary shortcoming of any co-culture system is the need to supply perhaps different needs for two different tissue types, often to the disadvantage of one or both tissues. In this system, conditions previously used for Leydig cell culture alone were adopted for co-culture of seminiferous tubules also. This suggests that since the requirements of these tissues are probably dissimilar, then one or both tissues will not be functioning optimally under these conditions. Secondly, the necessary disruption of the seminiferous tubules that must occur during their dissection may also affect their function in culture since the Sertoli cell tight junctions, which normally isolate the tubular epithelium from extra-

tubular factors, can perhaps now be bypassed via the cut ends of the seminiferous tubules. The relatively high levels of testosterone present in the isolated tubules(300-1,000pg/10 cm; see Chs 6-7) will also have had a confounding effect on the results, since during the culture period such testosterone as is present within the tubules is almost certain to diffuse into the medium. The confounding effect of this testosterone will have been more severe in conditions in which Leydig cell testosterone production was low. For example if Leydig cell testosterone production in the absence of hCG was 20 ng/million cells per 4 hours, the culture concentration of testosterone would be 4 ng/well. Thus testosterone from a 10 cm length of tubule could contribute up to 25% of the testosterone present in the culture medium. Such effects must be taken into consideration since the potential for falsely identifying 'Leydig cell stimulatory factors' is obvious. However in the presence of LH or hCG such errors are reduced dramatically since the Leydig cell contribution to the total testosterone present is correspondingly increased.

Despite these drawbacks to the use of co-culture systems other authors have reported positive findings. Parvinen and co-workers, using a similar system to the one described here reported stage-specific stimulation of Percoll-purified Leydig cell testosterone production by seminiferous tubules at stages VII-VIII of the spermatogenic cycle(Parvinen et al, 1984). However, in the same report, using unpurified Leydig cells these authors showed inhibition of testosterone production by tubules at all stages of the spermatogenic cycle. It would thus appear that purification of the Leydig cells affects their response to factor(s) produced by the seminiferous tubules. Parvinen's group used shorter incubation times than those

described for this study and the incubation medium also differed in that the only supplement used was 0.1% BSA, whilst the medium used for the present studies also contained insulin, transferrin, fungizone, ceruloplasmin, penicillin and streptomycin. The possibility of interference from one of these factors cannot be dismissed. The results discussed by Parvinen et al(1984) are supported by studies with porcine Sertoli-Leydig cell co-cultures(Tabone, Benahmed, Reventos & Saez, 1984), which demonstrated stimulation of steroidogenesis by porcine Leydig cells when co-cultured with porcine Sertoli cells. These authors also showed further stimulation of Leydig cell steroidogenesis when Sertoli cells were stimulated with FSH, suggesting a FSH-regulated mediator. However, since the Sertoli cells used for these studies were isolated from immature pigs, and Sertoli cell function is known to alter during development(see Chapter 1, or Sharpe, 1982 for review) the relevance of these results to the adult remains to be shown.

In summary, although it may be possible to demonstrate effects of seminiferous tubules upon Leydig cell steroidogenesis using a co-culture system, such effects are not easily reproducible in different laboratories and may be masked by the endogenous steroid content of the seminiferous tubules as well as by other factors. In these studies no effect of seminiferous tubules upon Leydig cells in co-culture could be demonstrated. Therefore as a means of circumventing these drawbacks a system of culturing Leydig cells in the presence of medium in which seminiferous tubules had previously been cultured was designed and tested(see Chapter 4).

CHAPTER 4

EFFECT OF SEMINIFEROUS TUBULE-CONDITIONED MEDIUM ON LEYDIG CELL TESTOSTERONE PRODUCTION

4.1: Introduction:

Studies on co-culture of seminiferous tubules and Leydig cells failed to establish whether the lack of detectable communication between seminiferous tubules and Leydig cells observed was physiologically relevant. It is possible to interpret the results from these co-cultures in two ways, as discussed above(see Chapter 3). Therefore, since it was not possible to study seminiferous tubule-Leydig cell communication using such a system, the decision was made to test a different system. By using seminiferous tubules to condition culture medium and thereafter exposing Leydig cells to this tissue-conditioned medium a number of novel possibilities are raised. Firstly, by extracting the steroids from the seminiferous tubule-conditioned medium it is possible to rule out interference by such factors and thereby the interpretation of results is simplified. Secondly, the production of such medium and the possibility of storing and accumulating quantities of conditioned medium allows the concentration and further investigation of the nature of any factor(s) identified by this procedure. With these aims in view, the following studies were undertaken.

4.2: Effect of seminiferous tubule-conditioned medium on unpurified Leydig cells:

Using medium conditioned with 10 cm/ml of adult rat seminiferous tubules or with strips of muscle tissue(see Chapter 2.5.1 for methods), co-cultured with unpurified Leydig cell preparations, no significant effect of conditioned medium upon Leydig cell testosterone production was evident, either basally or in the presence of a range of hCG concentrations(Fig 4.1).

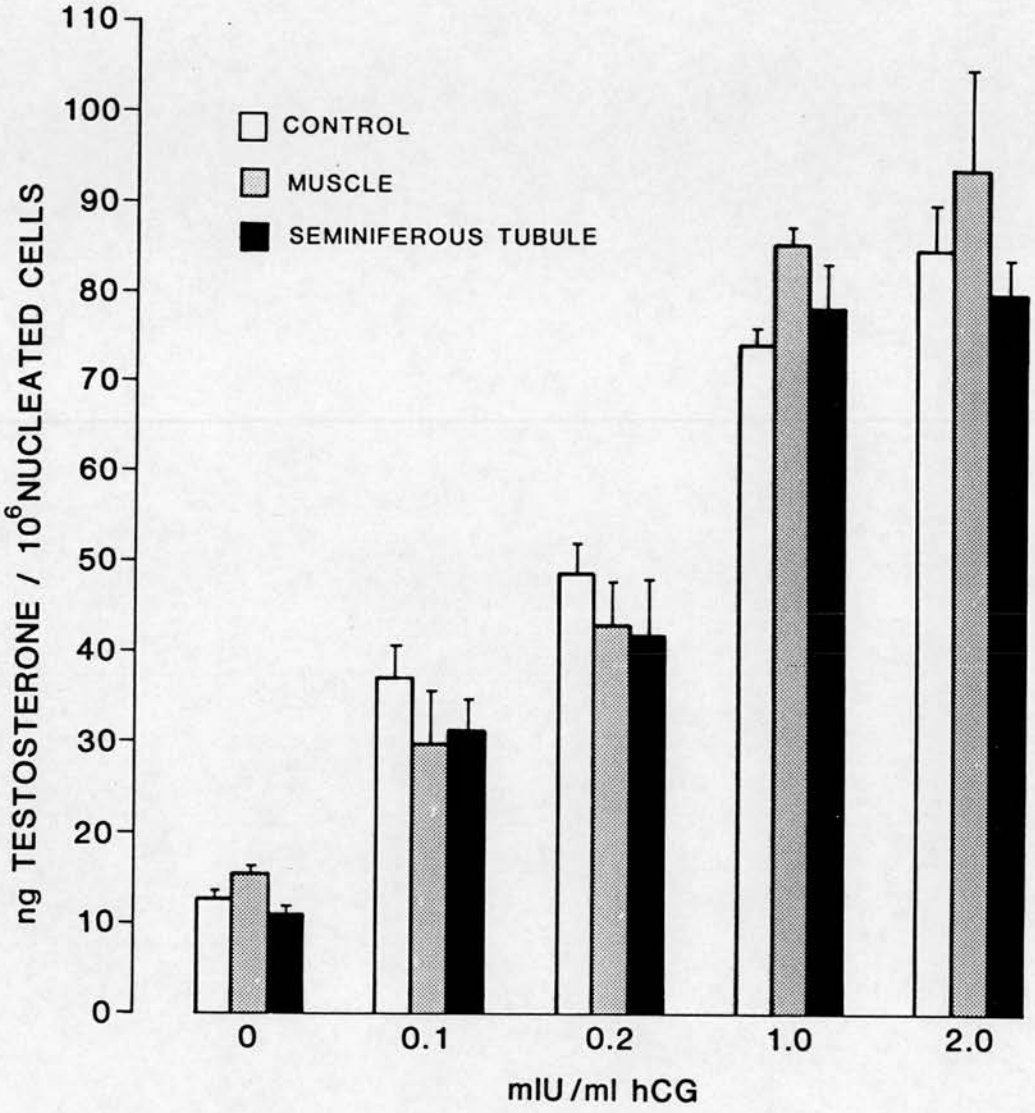


Fig 4.1: Effect of medium conditioned by isolated seminiferous tubules or muscle strips on testosterone production by unpurified Leydig cells during 5 h incubation in the presence of various doses of hCG(mean+/- s.d. of triplicate samples).

	TESTOSTERONE PRODUCTION ($ng/10^6$ CELLS)	
	BASAL	hCG(2 I.U./ml) STIMULATED
MEDIUM:		
Control	44+/-5	522+/-135
Muscle	50+/-11	480+/-80
S/T	68+/-13	585+/-107

Table 4.1: Effect of control, muscle-conditioned and seminiferous tubule(S/T)-conditioned media on testosterone production by Percoll purified Leydig cells during a 5 h incubation period. Controls represent Leydig cells alone(mean+/-s.d. of triplicate values).

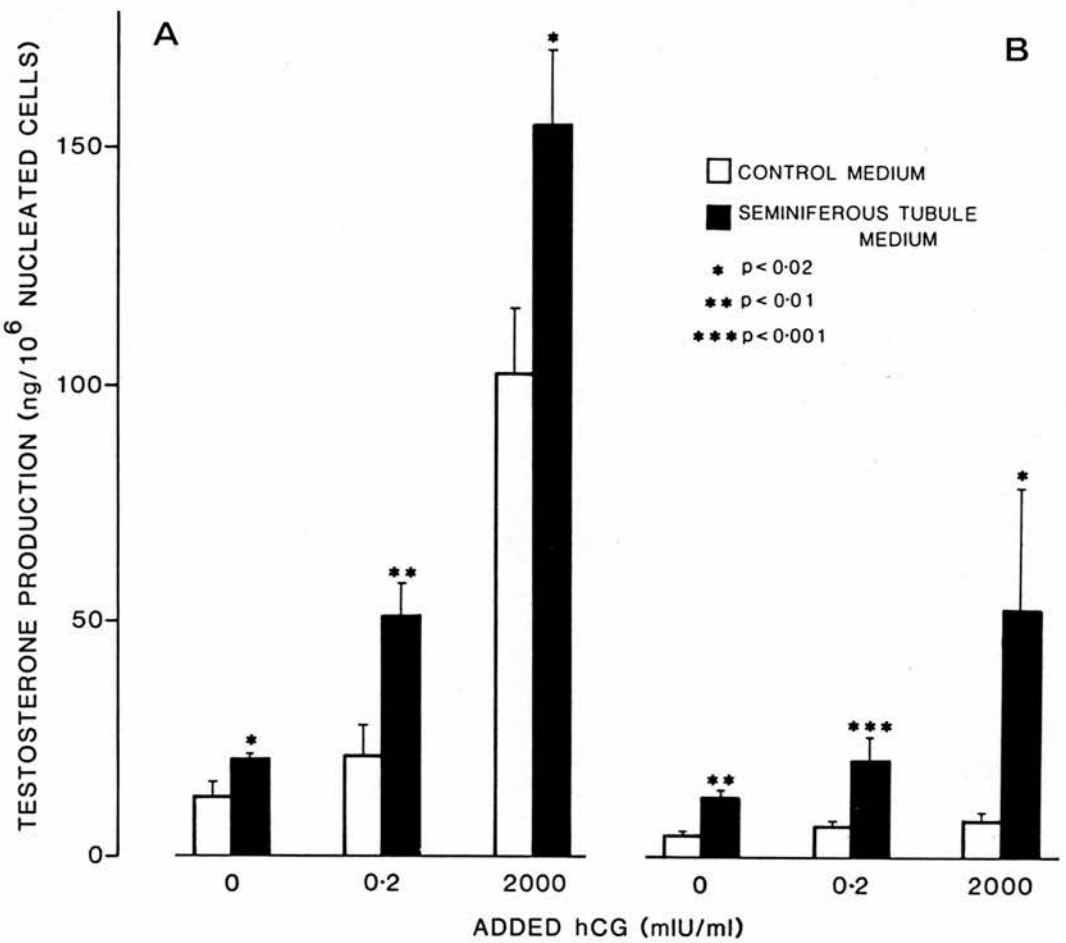


Fig 4.2: Effect of medium conditioned by isolated seminiferous tubules on testosterone production by Percoll purified Leydig cells during 5 h incubation either before(left) or after(right) a 24 h preincubation period. (Mean+/-s.d. of triplicate samples in both cases).

4.3: Effect of seminiferous tubule- or muscle-conditioned medium on Percoll-purified Leydig cells:

When muscle-conditioned medium was used as a tissue control no effect of such medium on basal or hCG-stimulated testosterone secretion by Leydig cells was found(Table 4.1). In the same experiment, increased testosterone secretion by Leydig cells co-incubated with seminiferous tubule-conditioned medium was seen.

During a subsequent experiment with a 5 h co-incubation of Leydig cells in seminiferous tubule-conditioned medium(10 cm/ml), a significant($p<0.01$) stimulation of Leydig cell testosterone production was observed both basally and in the presence of 0.2 and 2,000 mI.U./ml hCG(Fig 4.2.A). Seminiferous tubule-conditioned medium also significantly($p<0.001$) enhanced testosterone production by Leydig cells basally over 24 h(Control = 35.1+/-2.3ng testosterone/10^6 cells, Seminiferous tubule-conditioned = 44.4+/-4.3ng, $p<0.001$, $n=12$, mean+/-s.d.). During a subsequent 5 h incubation with 0, 0.2 or 2,000 mI.U./ml hCG no response of control Leydig cells to hCG was evident(Fig 4.2.B). However, Leydig cells incubated in seminiferous tubule-conditioned medium for the previous 24 h period retained some responsiveness to hCG stimulation, and produced significantly($p<0.02$-$p<0.001$) more testosterone than did their respective controls(Fig 4.2.B). However, due to the decrease in Leydig cell responsiveness, observed after a 24 h preincubation period, subsequent investigations were concentrated upon the initial 5 h of the incubation period.

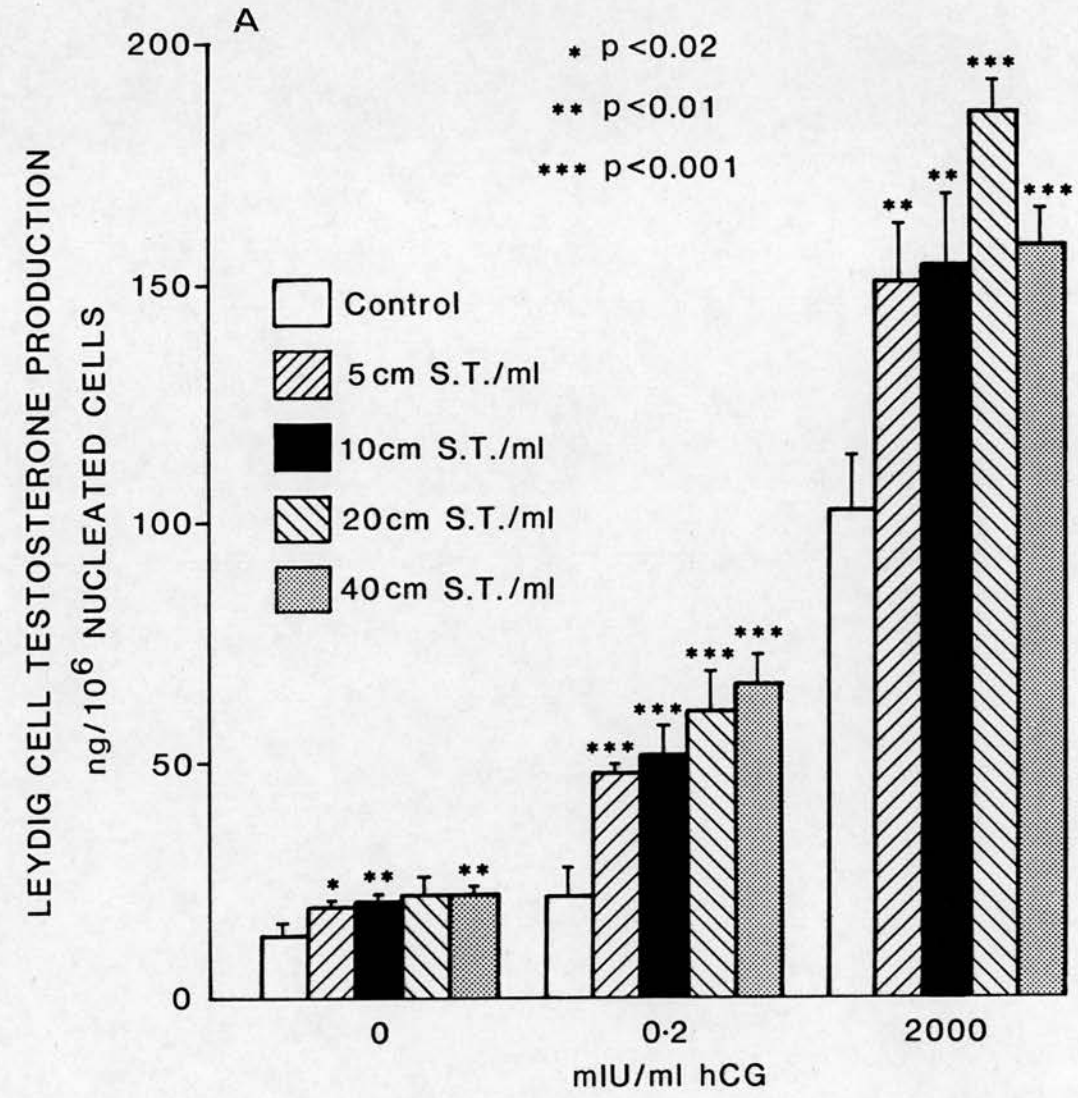
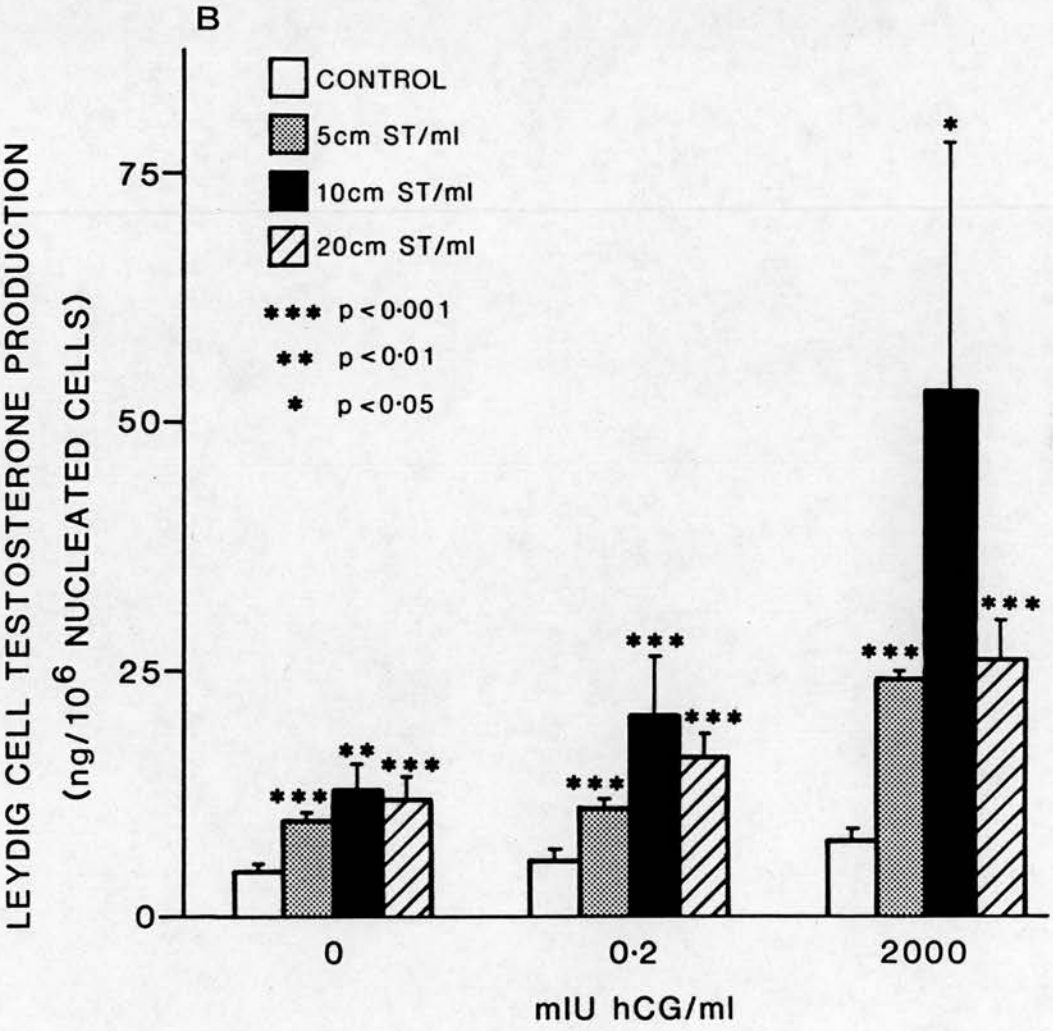


Fig 4.3: Effect of media conditioned by varying concentrations of isolated seminiferous tubules(5-40cm/ml) upon testosterone production by Percoll purified Leydig cells during 5 h incubation either before (above) or after(facing) a 24 h preincubation period(Mean+/-s.d. of quadruplicate samples in both cases).


B
CONTROL
5cm ST/ml
10cm ST/ml
20cm ST/ml
*** p < 0·001
** p < 0·01
* p < 0·05
LEYDIG CELL TESTOSTERONE PRODUCTION
(ng/10^6 NUCLEATED CELLS)
75
50
25
0
0
0·2
2000
mIU hCG/ml

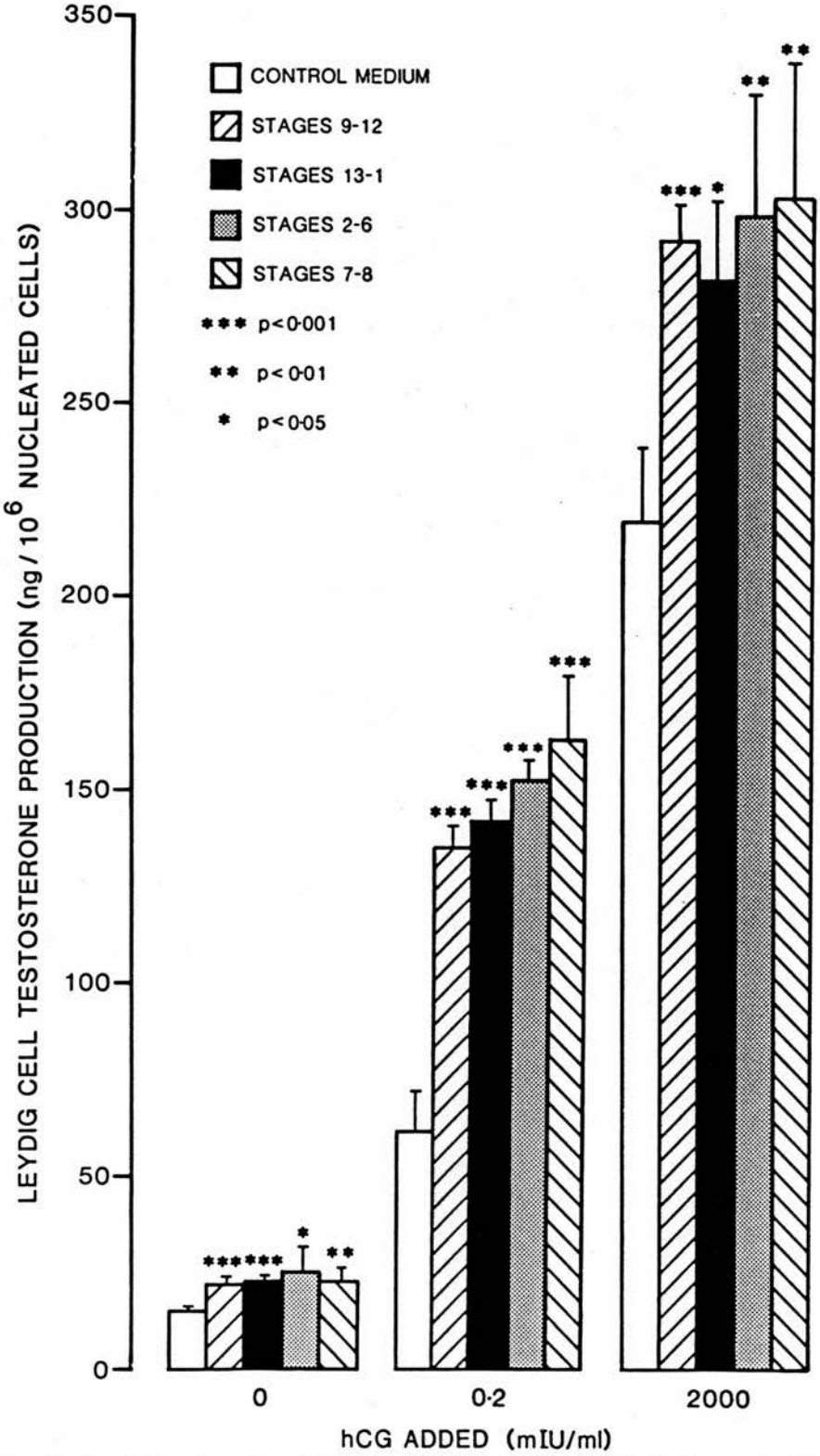


Fig 4.4: Effect of media conditioned by isolated seminiferous tubules from various stages of the spermatogenic cycle on testosterone production by Percoll purified Leydig cells during a 5 h incubation period(Mean+/-s.d. of quadruplicate values).

4.4: Effect of media conditioned by varying concentrations of isolated seminiferous tubules(5-40 cm/ml) upon testosterone production by Percoll-purified Leydig cells:

Medium prepared as described above which had been conditioned with differing amounts of seminiferous tubules/ml(i.e. 5 cm, 10cm, 20cm, and 40cm.) caused significant($p<0.02$-$p<0.001$) stimulation of both basal and hCG-stimulated testosterone production by Percoll-purified Leydig cells over 5 h, although there was no major difference in testosterone secretion caused by variation in the total lengths of seminiferous tubules used to condition the medium(Fig 4.3.A). However, following a 24 h preincubation in the presence of conditioned medium, and during a subsequent 5 h incubation with added hCG after renewal of the medium, the media conditioned by 10 cm and 20 cm tubules/ml resulted in significantly($p<0.01$) greater enhancement of testosterone production than did the 5 cm/ml group(40 cm/ml group not tested, Fig 4.3.B). Whilst the difference between the 10 cm/ml and 20 cm/ml media was not significant, in view of the difference in the time required to prepare these media it was considered more practical to adopt 10 cm/ml of seminiferous tubules as the optimum level for the production of conditioned medium in subsequent experiments.

4.5: Stage-dependent effects:

Conditioned medium was prepared using seminiferous tubules from different stages of the spermatogenic cycle(Stages II-VI, VII-VIII, IX-XII and XIII-I). Seminiferous tubule-conditioned medium from all groups of stages significantly enhanced basal and hCG-stimulated testosterone production by Leydig cells during a 5 h incubation (Fig 4.4). This experiment was repeated and results similar to those

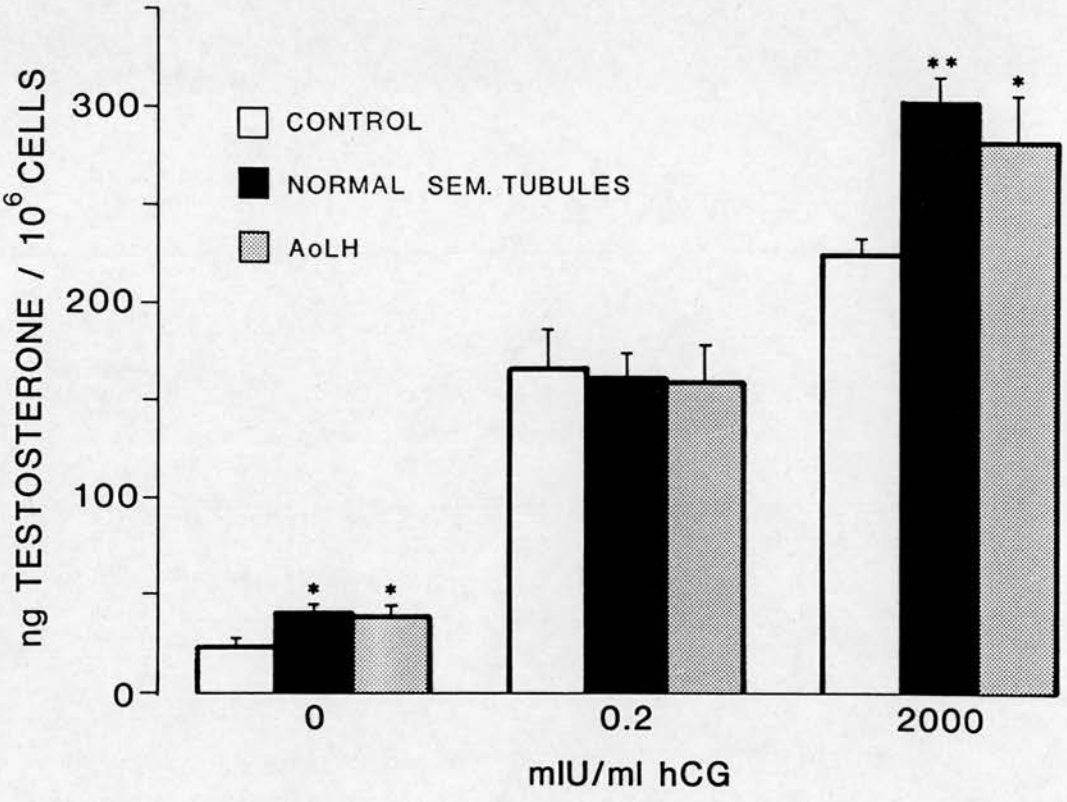


Fig 4.5: Effect of media conditioned by isolated seminiferous tubules from animals treated 40 h previously with an antiserum to oLH on testosterone production by Percoll purified Leydig cells during a 5 h incubation period(mean+/-s.d. of quadruplicate values) * $p<0.05$, ** $p<0.01$ compared with control values.

illustrated(Fig 4.4) were achieved i.e. whilst all stages significantly enhanced Leydig cell testosterone production, no significant variation was seen in the ability of medium conditioned by seminiferous tubules at different stages to enhance Leydig cell testosterone secretion(Fig 4.4).

4.6: Effects of different in vivo treatments:

The results described above allow two possible interpretations. They may be caused either by the release of non-specific factors which improve the function of Leydig cells in culture, or they may reflect the production of specific factors, with physiological roles, which modify Leydig cell testosterone production. In an attempt to discriminate between these two possibilities the effects of hormone deprivation, using an antiserum to LH, and the effects of hypophysectomy and cryptorchidism upon the ability of seminiferous tubule-conditioned medium to modify Leydig cell testosterone production were investigated.

4.6.1: Effects of an LH antiserum:

Using medium conditioned by tubules from normal adult rats and from rats injected 40 h beforehand with anti-LH the effects of LH and therefore testosterone deprivation upon the secretion or release of seminiferous tubule factor(s) was investigated. Seminiferous tubule-conditioned medium from both normal and anti-LH treated rats significantly($p<0.01$-$p<0.001$) increased Leydig cell testosterone secretion above control values in the presence of maximal(2,000 mI.U./ml) hCG stimulation and also in the absence of any added gonadotrophin stimulus(Fig 4.5). There was no difference in the degree of stimulation of Leydig cell testosterone production induced by medium conditioned with seminiferous tubules from control and anti-LH

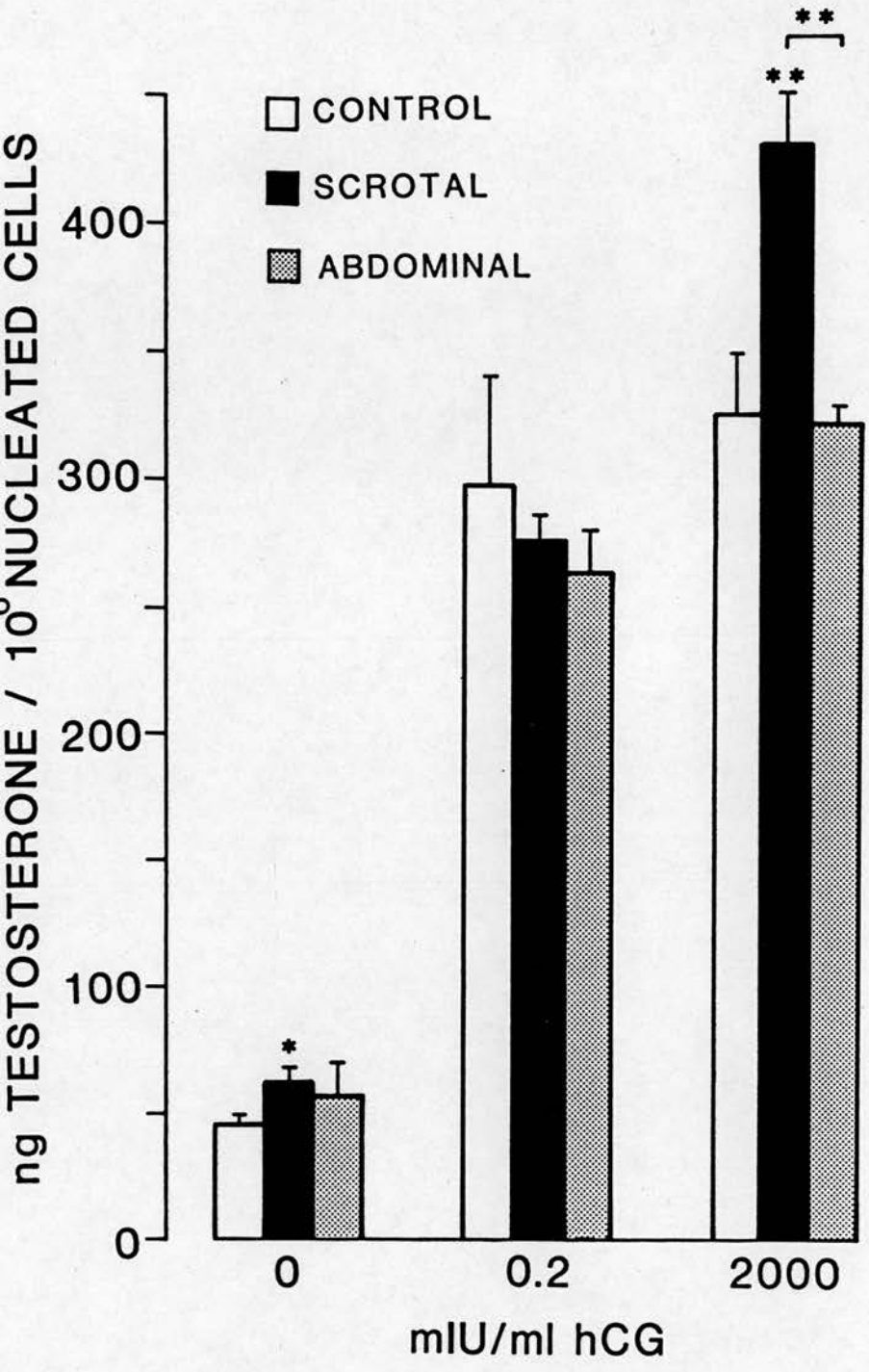


Fig 4.6: Effect of media conditioned by isolated seminiferous tubules from animals made unilaterally cryptorchid(see text) on testosterone production by Percoll purified Leydig cells during a 5 h incubation period (mean+/-s.d. of quadruplicate values) * $p<0.05$, ** $p<0.01$ compared with either control or bracketed values.

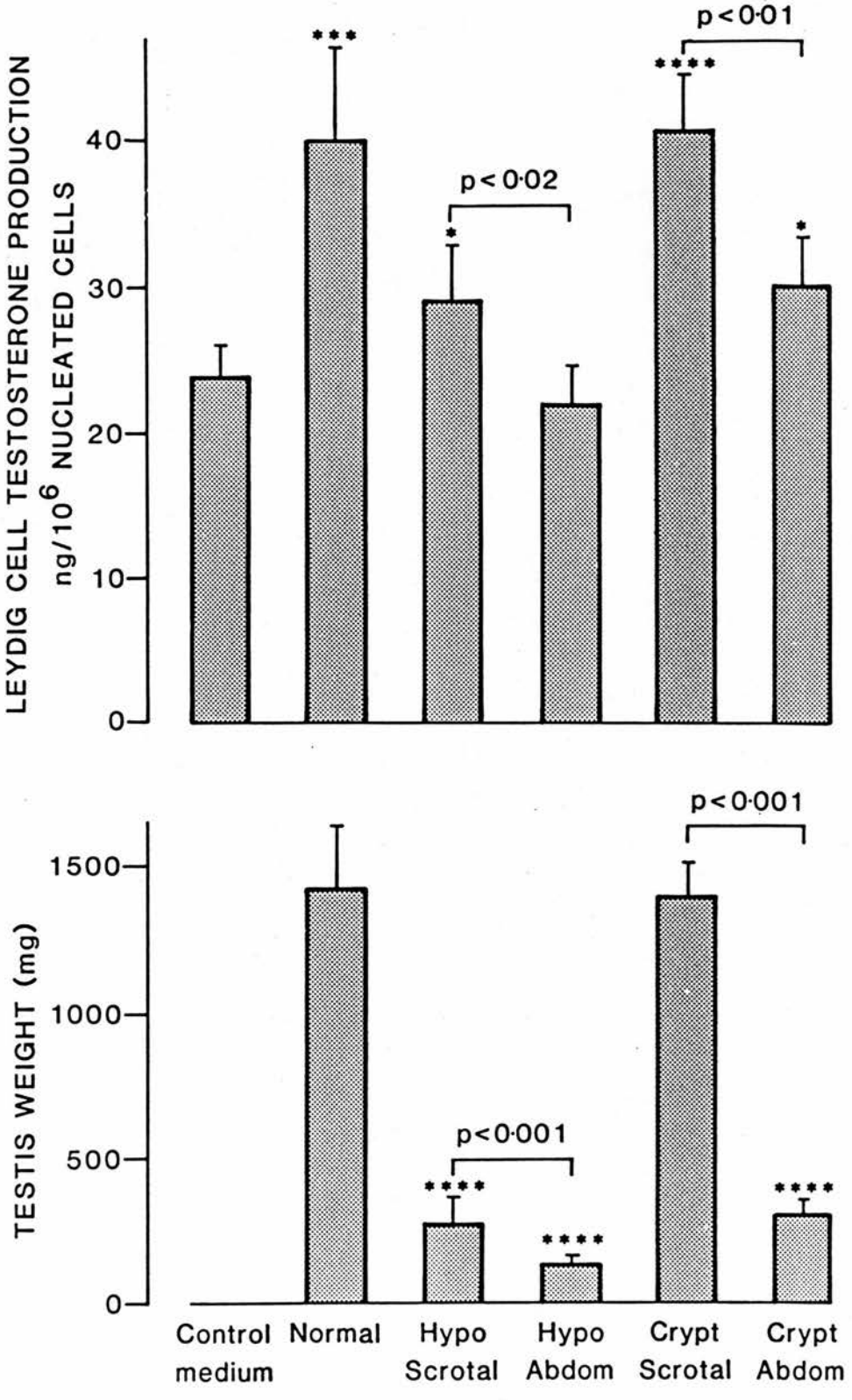

LEYDIG CELL TESTOSTERONE PRODUCTION
ng/10^6 NUCLEATED CELLS
0
10
20
30
40
p<0·01
p<0·02
TESTIS WEIGHT (mg)
0
500
1000
1500
p<0·001
p<0·001
Control medium
Normal
Hypo Scrotal
Hypo Abdom
Crypt Scrotal
Crypt Abdom

Fig 4.7: Effect of media conditioned by isolated seminiferous tubules from rats subjected to hypophysectomy, unilateral cryptorchidism or both treatments simultaneously, on testosterone secretion by Percoll purified Leydig cells in the absence of added hCG stimulation(Top panel) during a 5 h incubation period(mean+/-s.d. of quadruplicate values). The bottom panel shows the testis weights of the animals from which the tubules were dissected. * $p<0.05$, *** $p<0.01$, **** $p<0.001$ compared with control values. Other significance values refer to differences between bracketed groups.

treated rats. No effect of either batch of tubule-conditioned medium was seen at a sub-maximal(0.2 mI.U./ml) dose of hCG stimulation.

4.6.2: Effects of long term cryptorchidism:

When medium conditioned with seminiferous tubules from the abdominal testes of rats which had been made unilaterally cryptorchid (UCD) 95 days previously was added to Leydig cells, no stimulatory effects of such medium upon Leydig cell steroidogenesis were seen. Medium conditioned with control(scrotal) seminiferous tubules, screened in the same experiment, significantly ($p<0.01$-$p<0.001$) increased basal and hCG-stimulated(2,000 mI.U./ml hCG) Leydig cell testosterone production(Fig 4.6). No effect of medium prepared from tubules of either the cryptorchid or control testes upon submaximally stimulated(0.2 mI.U./ml hCG) Leydig cell testosterone production was seen.

4.6.3: Effects of hypophysectomy, cryptorchidism and combined hypophysectomy and cryptorchidism:

To assess the possible physiological significance of the described effects, seminiferous tubule-conditioned medium was prepared using tubules from testes in which depletion of the germ cells was achieved by unilateral cryptorchidism, hypophysectomy, or a combination of these procedures. Rats were hypophysectomized on day 52 after birth, made unilaterally cryptorchid 3 days later, and eventually killed at 79 days of age. Intact male rats were made unilaterally cryptorchid at 86 days of age and killed 42 days later. Medium conditioned by seminiferous tubules from normal control rats or by tubules from the scrotal testes of unilaterally cryptorchid rats significantly stimulated basal testosterone production by Leydig cells($p<0.01$ and $p<0.001$, respectively; Fig 4.7). Medium conditioned

by seminiferous tubules from the abdominal testes of unilaterally cryptorchid rats and from the scrotal testes of unilaterally cryptorchid hypophysectomized rats also significantly stimulated Leydig cell testosterone production($p<0.05$) but to a significantly ($p<0.01$) lesser extent than did either medium conditioned with seminiferous tubules from normal rats or from scrotal testes of unilaterally cryptorchid rats(Fig 4.7). Medium conditioned by seminiferous tubules from the abdominal testes of unilaterally cryptorchid hypophysectomized animals induced no increase whatsoever in testosterone production by Leydig cells. The degree of seminiferous tubule 'damage' induced by these various conditions, as assessed by testicular weights for each of the treatments, closely correlated with the degree of stimulation of Leydig cells by seminiferous tubule-conditioned medium as described above(Fig 4.7). However, no effect of the conditioned media from any of the groups of animals was seen on Leydig cells which were either maximally (2,000 mI.U./ml hCG) or sub-maximally (0.2 mI.U./ml hCG)stimulated with hCG(data not shown).

4.7: Assessment of the reproducibility of results:

The results described above(See 4.6.2-3) in which no effect of tubule-conditioned medium was seen under some experimental conditions calls into question the consistency and reproducibility of the stimulatory effects of seminiferous tubule-conditioned medium observed. In further studies, undertaken to determine the reliability of the methods described here, the effect of medium conditioned during 20-24 h incubation with 10cm/ml seminiferous tubules, on basal and hCG stimulated testosterone production by Percoll-purified Leydig cells was assessed(Table 4.2). In 13 consecutive experiments seminiferous

EXPT No:	TESTOSTERONE PRODUCTION ($NG/10^6$ CELLS) BASAL		hCG (2 IU/ml) STIMULATION	
	CONTROL	S/T	CONTROL	S/T
1	22.8+/-1	23.1+/-1	282+/-27	302+/-12
2	46.7+/-5	62.0+/-7*	437+/-76	380+/-37
3	47.0+/-8	47.6+/-8	250+/-15	308+/-31*
4	20.8+/-2	26.0+/-2*	157+/-12	184+/-4*
5	46.4+/-2	61.7+/-3**	325+/-20	431+/-18***
6	52.2+/-3	58.4+/-6	366+/-14	393+/-22
7	23.6+/-4	41.7+/-5**	223+/-8	303+/-11***
8	40.6+/-7	82.6+/-15**	358+/-21	393+/-43
9	23.8+/-2	40.2+/-5**	258+/-19	277+/-9
10	22.3+/-2	63.6+/-7***	140+/-19	196+/-12**
11	26.6+/-6	41.1+/-1**	204+/-24	306+/-31**
12	15.0+/-1	22.2+/-2***	220+/-19	292+/-9***
13	23.9+/-2	32.5+/-4**	246+/-6	260+/-18

Table 4.2: Reproducibility of the effect of seminiferous tubule-conditioned medium(S/T) on Leydig cell testosterone production by Percoll purified Leydig cells during 5h incubation(Mean+/-s.d. n=4). * Significant differences between controls and S/T exposed Leydig cells(* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

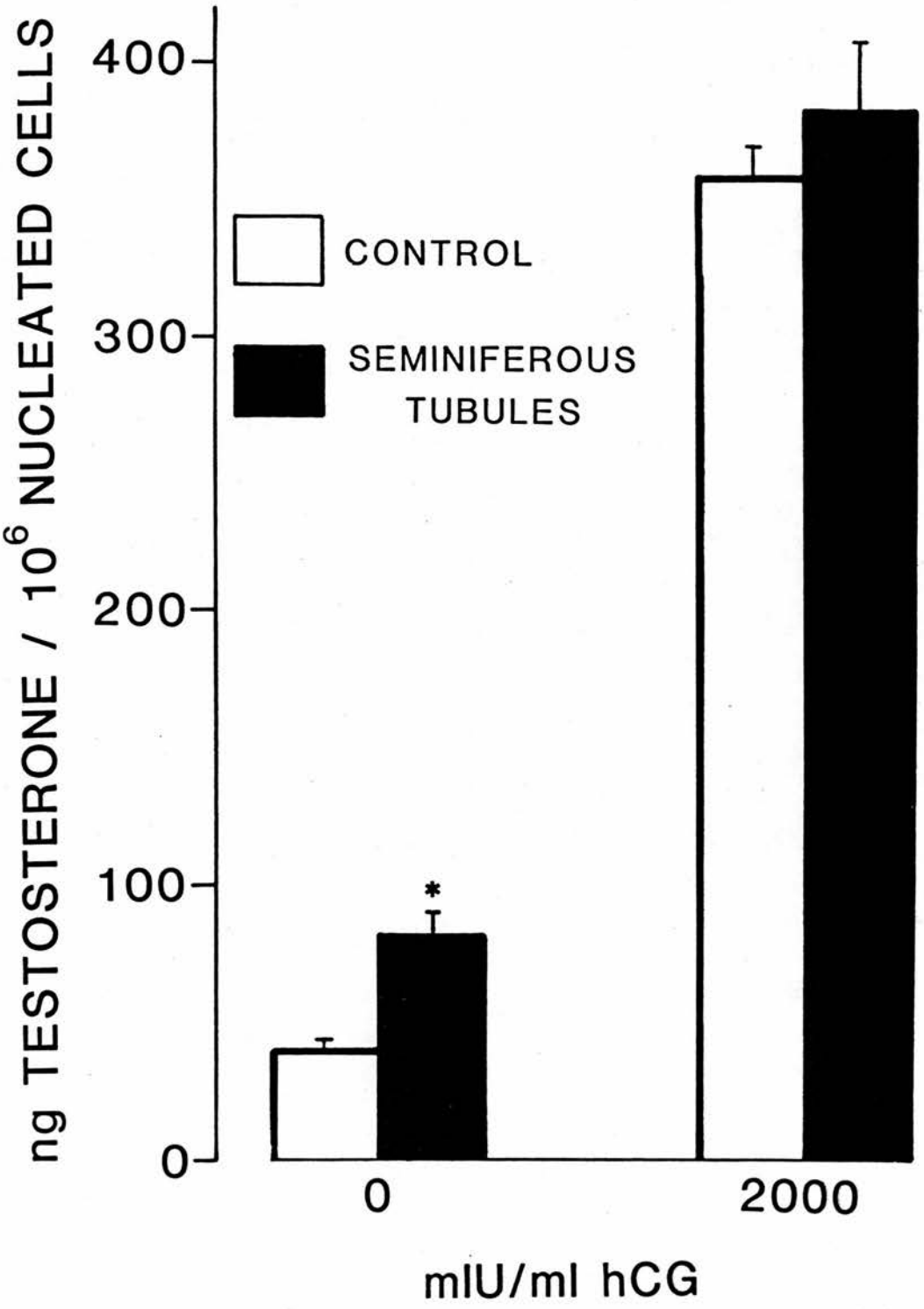


Fig 4.8: Effect of dialysis on the ability of seminiferous tubule-conditioned medium to stimulate testosterone production by Percoll purified Leydig cells during a 5h incubation period(mean+/-s.d. of quadruplicate values). * $p<0.05$ when compared with control medium.

tubule-conditioned medium stimulated basal testosterone production by Leydig cells in 11/13 experiments and in 10 out of the 13 experiments this difference was statistically significant($p<0.02$-$p<0.001$). Similarly, testosterone production in response to hCG was enhanced in all but one experiment, although this reached significance in only 7 of the 13 experiments($p<0.02$-$p<0.001$)(Table 4.2). In this study there was considerable variation between experiments in the amounts of testosterone secreted basally and in response to hCG and this may reflect differences in the percentage of Leydig cells in the different cell preparations or differences in their responsiveness. However, there was no consistent relationship between these variables and the degree of stimulation of testosterone production elicited by seminiferous tubule-conditioned medium(Table 4.2).

4.8: Concentration of seminiferous tubule-conditioned medium:

In view of this inconsistency, and also to determine whether further studies upon the nature and physiology of the seminiferous tubule factor(s) were feasible, attempts were made to concentrate the active component of the conditioned medium using ammonium sulphate precipitation.

4.8.1: Effects of dialysis:

As a preliminary to detailed experiments on ammonium sulphate precipitation 10 mls of seminiferous tubule-conditioned and control medium were dialysed overnight against M199H. The dialysis membrane had a molecular cut-off of about 10,000 daltons. The results shown (Fig 4.8) suggest that dialysis would be a suitable desalting procedure following ammonium sulphate precipitation, and incidentally that the active seminiferous tubule factor(s) has a molecular weight in excess of 10,000 daltons.

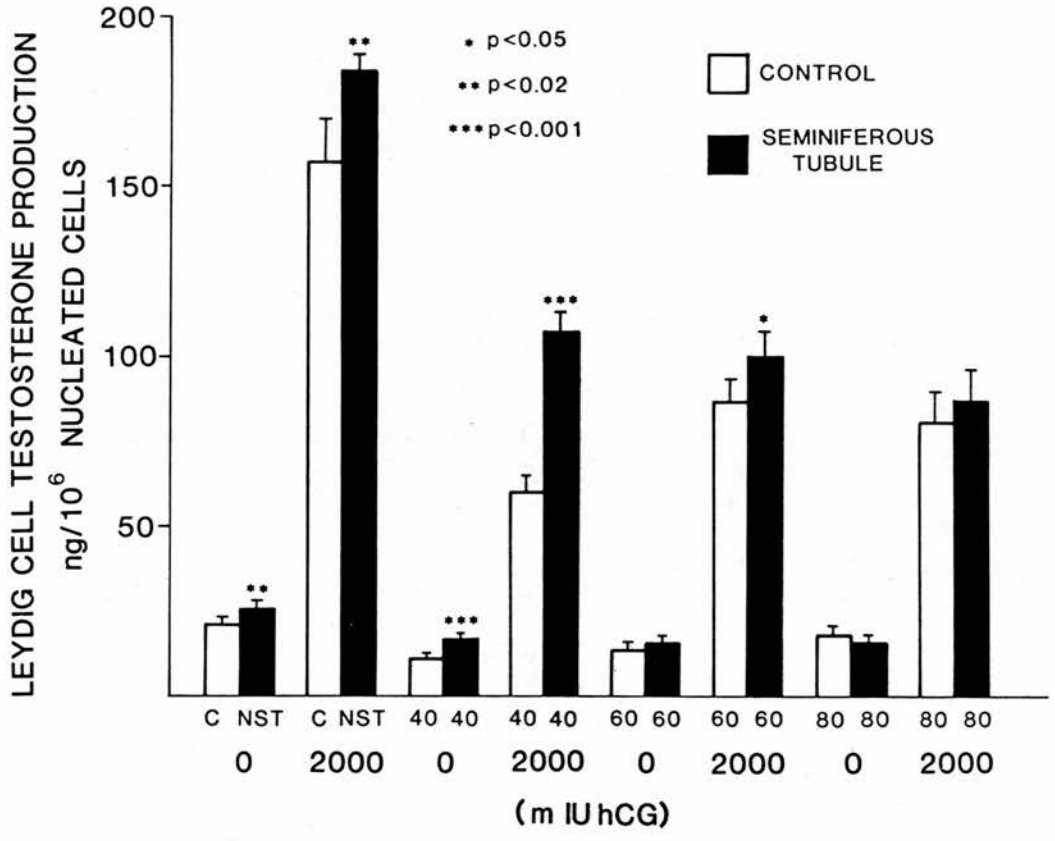


Fig 4.9: Effect of ammonium sulphate precipitates of M199E(control) or seminiferous tubule-conditioned medium on testosterone production by Percoll purified Leydig cells during a 5 h incubation period (mean+/-s.d. of quadruplicate values). C = control medium untreated, NST = untreated seminiferous tubule conditioned medium. 40 = 40% ammonium sulphate precipitate of control(open bar) or seminiferous tubule conditioned medium(solid bar). 60 = 60% ammonium sulphate precipitate, 80 = 80% ammonium sulpate precipitate. Significances refer to comparisons with the relative control values.

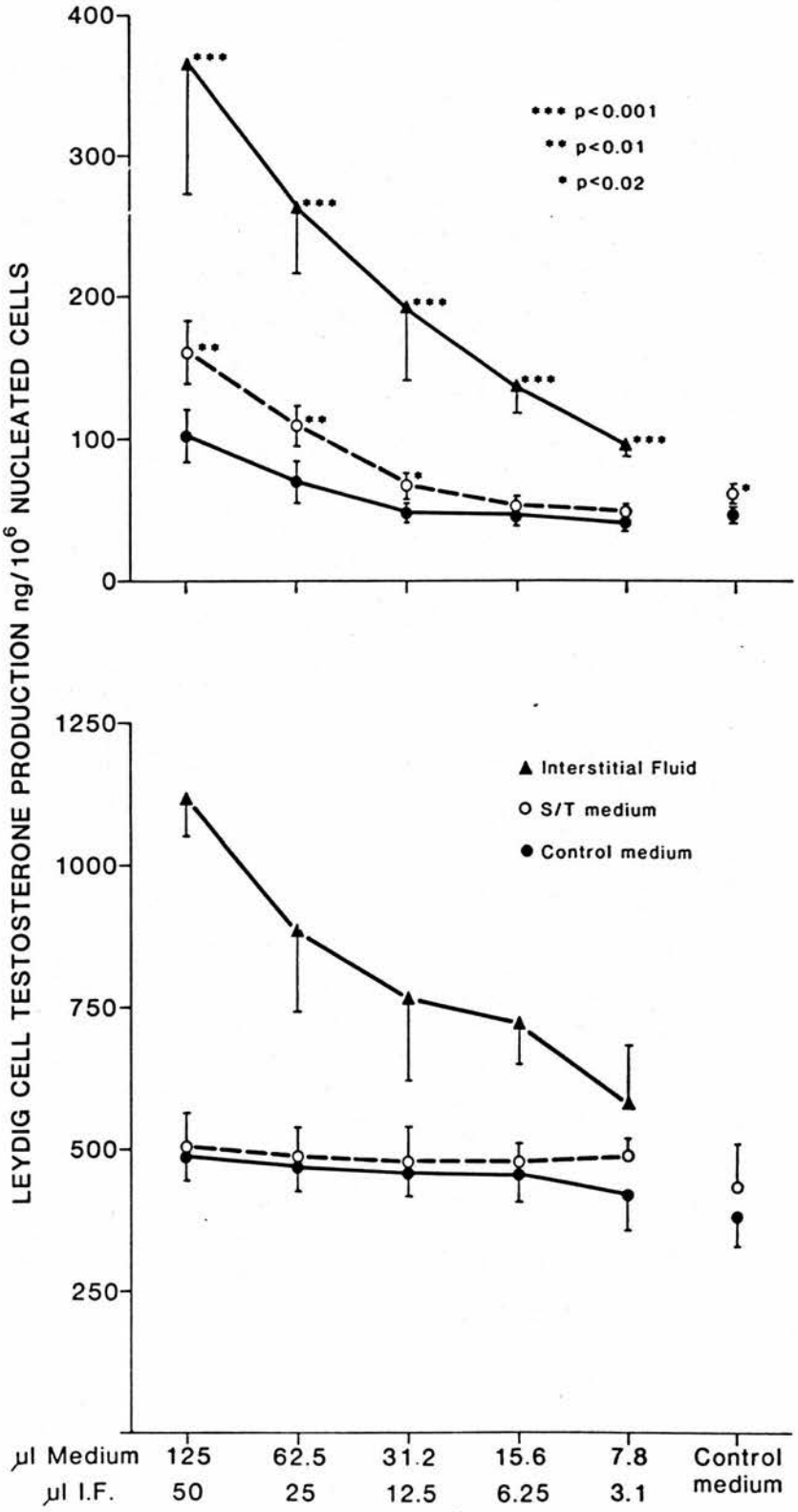


Fig 4.10: Double dilution of 60% ammonium sulphate precipitate of control and seminiferous tubule conditioned(S/T) media, compared with charcoal stripped interstitial fluid(IF) and untreated medium. The effect of the addition of these factors on testosterone production by Percoll purified Leydig cells during a 5 h incubation period(mean+/- s.d. of quadruplicate values) in the absence of added hCG(top) or with 2 mI.U./ml hCG added(bottom) is shown.

4.8.2: Precipitation of proteins with ammonium sulphate:

a) Experiment 1:

Stepwise precipitation of 20 mls of seminiferous tubule-conditioned medium with increasing concentrations of ammonium sulphate concentrated the factor(s) shown to increase Leydig cell testosterone production in the 40% ammonium sulphate precipitate(Fig 4.9). However, all of the precipitates from both control and seminiferous tubule-conditioned media, decreased Leydig cell responsiveness to hCG, possibly due to inadequate desalting or to high protein concentrations.

b) Experiment 2:

100 mls of seminiferous tubule-conditioned and control media were stepwise precipitated with ammonium sulphate and, after desalting by dialysis, the most active fraction was identified by a similar procedure to that shown above(4.8.2.A). The Leydig cell stimulatory activity in these fractions was concentrated in the 60% ammonium sulphate precipitate. This precipitate was then used to provide a comparison between the activity of the seminiferous tubule factor(s) and a factor(s) recently described in testicular interstitial fluid(Sharpe and Cooper, 1984) which also has marked stimulatory effects on the testosterone production of Percoll purified Leydig cells and is precipitated by 60% ammonium sulphate. Both rat interstitial fluid and the medium precipitates were double diluted and aliquots from each dilution incubated with Percoll purified Leydig cells in the presence of maximally stimulating concentrations of hCG (2,000 mI.U/ml) or in the absence of added hCG. The results(Fig 4.10.A) showed that in the absence of hCG the seminiferous tubule conditioned medium showed stimulatory activity with doses ranging from

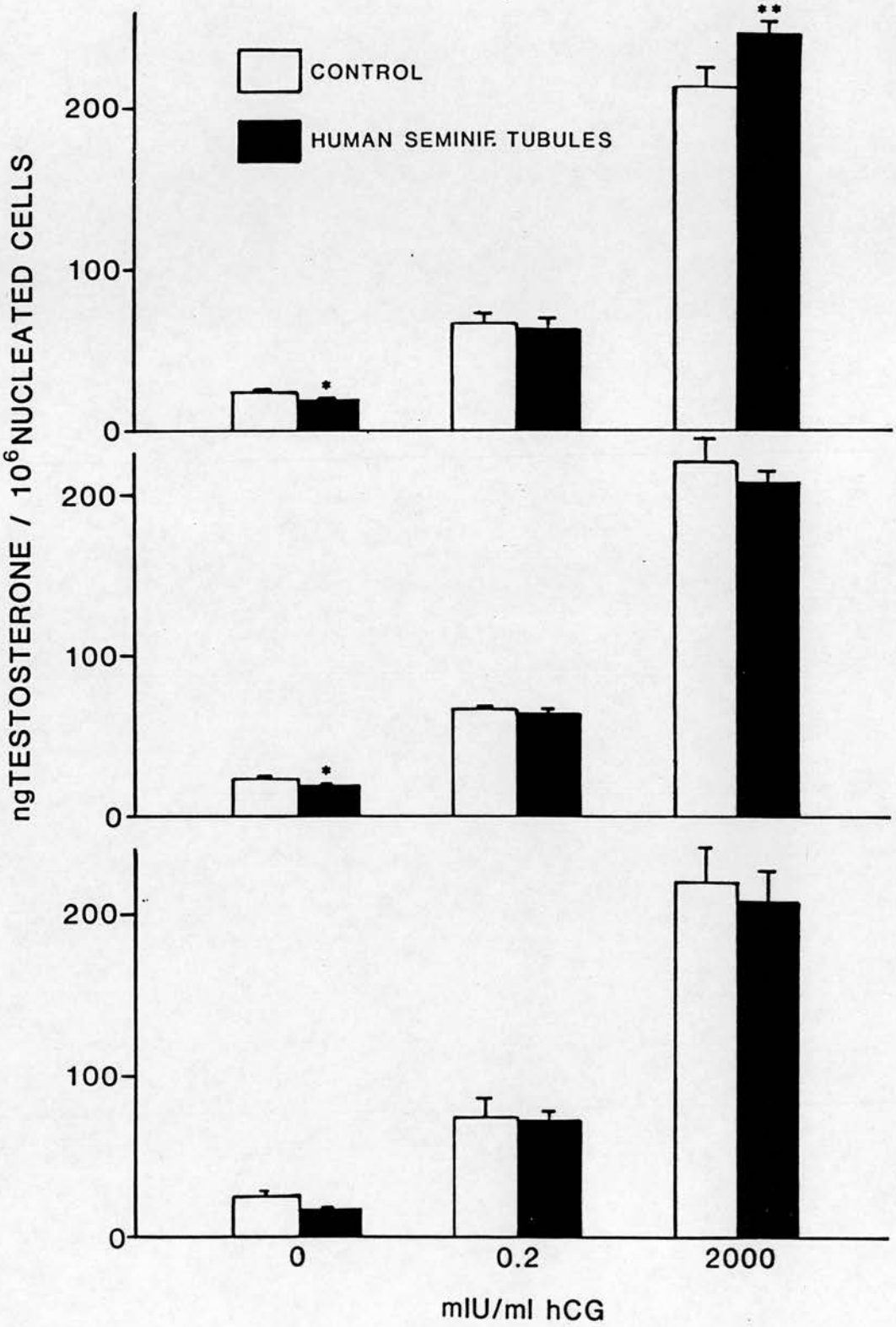


Fig 4.11: Effect of media conditioned by isolated seminiferous tubules from three human testes on testosterone production by Percoll purified rat Leydig cells during a 5 h incubation period(mean+/-s.d. quadruplicate values). * $p<0.05$, ** $p<0.01$ when compared with control medium.

125-31.2 µl of medium per well. The interstitial fluid also caused a dose-related stimulation of testosterone production by Leydig cells over the range 50-3.1 µl/well. The increased activity shown by the concentrated seminiferous tubule medium represented an 8 fold increase over control values(i.e. 8 times as much untreated conditioned medium would be required to give the same effect as the treated medium). However, in the presence of maximally-stimulating concentrations of hCG, no effect of the concentrated seminiferous tubule-conditioned medium was seen(Fig 4.10.B), although interstitial fluid still markedly enhanced hCG-stimulated Leydig cell testosterone production.

4.9: Effects of medium conditioned by human seminiferous tubules:

To assess the potential for the screening of human tissue in this system, medium was prepared from three patients who underwent sub-capsular orchidectomy as a treatment for prostatic cancer. Seminiferous tubules from the three men, aged 70(Fig 4.11.A), 80(Fig 4.11.B) and 58(4.11.C) years were prepared in an identical manner to rat seminiferous tubules. Each of the patients gave differing results (Fig 4.11). Medium conditioned by seminiferous tubules from two of the three patients caused a significant($p<0.05$) decrease in basal Leydig cell testosterone production when compared with control medium(Fig 4.11.A-B), whilst those from a third caused a non-significant decrease. Medium prepared with seminiferous tubules from one patient (Fig 4.11.C) significantly enhanced maximal hCG-stimulated Leydig cell steroidogenesis($p<0.01$) although no effect of medium conditioned with tubules from the other two patients was observed, nor was any effect of medium conditioned with human seminiferous tubules seen at a dose of hCG(0.2 mI.U./ml) considered to produce sub-maximal stimulation of Leydig cell steroidogenesis(Fig 4.11).

4.10: Discussion:

The purpose of this study was to set up a suitable in vitro system to investigate cell-cell interactions between Leydig cells and seminiferous tubules. Whilst the results presented show that a factor(s) is released or secreted from the seminiferous tubules which has a stimulatory effect on testosterone production by Percoll-purified Leydig cells both in the presence and absence of gonadotrophins, these findings were somewhat inconsistent. Therefore, although the findings presented add further support to the growing evidence suggesting that seminiferous tubules exert a stimulatory influence on the Leydig cells, the inconsistency of these findings gives cause for concern. There are several possible explanations for this lack of consistency:

Firstly, the obvious advantage of using seminiferous tubule-conditioned medium was that seminiferous tubules could be incubated for up to 24 h, during which time levels of secreted factors could accumulate prior to incubation of this conditioned medium with Leydig cells. However, the integrity and viability of the tubules may have varied from preparation to preparation, due to differences in the extent of collagenase dispersion to which they had been exposed. Over-digestion of testes causes the tubules to become fragile and friable during the dissection and isolation procedures and this may also mirror their viability in culture. Initial results of histological examinations suggested some damage to tubules centered on the nuclei of type II spermatocytes, and this was evident within 30 min of culture but remained constant, i.e. did not progress thereafter. Damage incurred during the preparation of tubules could affect their subsequent ability to produce and release factors able to affect the

Leydig cells.

A further factor affecting the results may be the variability in responsiveness of the isolated Leydig cells. Studies showing that the isolation procedure can radically affect the ability of Leydig cells to secrete testosterone in culture(Aldred & Cooke, 1983; Sharpe & Cooper; 1982, Molenaar et al, 1983) suggest that these cells are very sensitive to damage during isolation. Additionally, in our laboratory only about 30% of Leydig cell preparations respond to LHRH-agonist in the presence of maximally-stimulating concentrations of hCG, whilst all cell preparations respond to LHRH-agonist in the absence of added hCG(Sharpe, unpublished data). Whilst there is no explanation for this observation, it is possible that a similar variability may explain why over 77% of preparations of tubule-conditioned medium stimulated basal Leydig cell testosterone secretion significantly, whilst hCG-stimulated testosterone secretion was enhanced significantly by only 57% of the preparations.

Moreover, the factor present in seminiferous tubule-conditioned medium may not be particularly stable, contributing to the variability of results. This supposition is supported by the likelihood that local factors produced within the testis will be degraded rapidly because of the nature of their function, i.e. because they must only act locally. It has been shown that the testicular interstitial fluid factor mentioned above loses activity readily under certain conditions, e.g. after lyophilization(G. Risbridger & R.M. Sharpe, unpublished data), and this is also true for the seminiferous tubule-conditioned medium described here(data not shown).

Finally it is possible that the material tested here represents not just a single active factor but two or more factors capable of

acting at different hCG/LH concentrations in different ways. There is adequate support within the literature for the presence of a number of different intratesticular factors, for example 'testicular-LHRH'(Sharpe, 1982 for review), a testicular interstitial fluid factor(Sharpe & Cooper, 1984), and a number of reports of factors present in Sertoli cell-conditioned medium or of stimulatory effects on Leydig cells when co-cultured with seminiferous tubules(Syed, Khan & Ritzen, 1985; Grotjans & Heindel, 1982; Verhoeven & Cailleau, 1984; Sharpe & Rommerts, 1984; Parvinen et al, 1984). However, none of these factors are well characterised and it therefore remains unlikely that, at the present time, such factors could be identified individually when more than one may be present in the same test system.

These studies failed to show any effect of seminiferous tubule-conditioned medium upon unpurified collagenase-dispersed rat interstitial cells. However, recent studies by Syed et al,(1985) have shown that when spent medium from stage-dissected seminiferous tubules was incubated with unpurified rat interstitial cells a stage-specific inhibition of testosterone production occurred with medium conditioned by tubules at stages VII-VIII. The discrepancy between these studies could be due to a number of differences between the techniques: Firstly, Syed et al used stage dependent dissections of seminiferous tubules to show effects on Leydig cell testosterone production, while the results described above using unpurified Leydig cells made use of randomly dissected tubules. Syed et al(1985) showed that while stages VII-VIII inhibited Leydig cell testosterone production, no effect upon unpurified interstitial cell testosterone production was seen at other stages. Therefore, it is likely that

using random tubules the specific effects seen at stages VII-VIII would be 'diluted out'. Secondly, Syed et al used 20 cm/ml of tubules to condition the medium, twice the tissue concentration used above. Finally, whilst the present studies used charcoal-stripped seminiferous tubule-conditioned medium, Syed et al(1985) used whole medium without stripping with charcoal and it is possible that some small molecular weight factors were removed by charcoal stripping of medium.

Despite the lack of an effect of tubule-conditioned medium upon testosterone production by unpurified interstitial cells, Percoll-purified cells showed a significant increase in testosterone production in the presence of tubule conditioned medium. This effect was not seen with muscle conditioned medium, used as a control tissue. These results are in agreement with results previously published by a number of groups on stimulatory effects of Sertoli cell-conditioned medium(Grotjans & Heindel, 1982; Verhoeven & Cailleau, 1984; Sharpe & Rommerts, 1984), seminiferous tubule-conditioned medium(Syed et al, 1985), pig Sertoli-Leydig cell co-cultures(Tabone et al, 1984) and rat seminiferous tubule-Leydig cell co-cultures(Parvinen et al, 1984). It is clear from these studies and those reported above that there exist within the testis factors which can markedly affect Leydig cell testosterone production. One such factor, a testicular LHRH-like peptide has been identified previously(Sharpe et al, 1984 for review), and shown to have direct effects upon the testis(Huhtaniemi et al, 1985). Recently the presence of a factor which markedly stimulates Leydig cell testosterone production, either in the absence or in the presence of maximal doses of hCG, has been reported(Sharpe & Cooper, 1984). This interstitial fluid factor has similar effects to that of

the seminiferous tubule-conditioned medium reported above, although its effect is markedly greater. It is becoming increasingly apparent that the control of Leydig cell steroidogenesis relies on both peripheral and local hormonal stimuli and that the local factors controlling this aspect of testicular function may be numerous and show diverse activities.

Significant stimulation of Leydig cell testosterone secretion was seen with media conditioned by seminiferous tubules from all stages of the spermatogenic cycle. However, although stages VII-VIII gave a consistently higher level of stimulation than did other stages, this did not reach significance. Therefore, although this trend may reflect a real difference in the levels of the active factor(s) released from seminiferous tubules at different stages of the cycle, as has been suggested by recent findings(Parvinen et al, 1984; Syed et al, 1985), the system described here was not sufficiently sensitive to pick up these changes. Such a stage-related change in secretion of this factor would support evidence from other groups that tubules at stages VII-VIII have a greater requirement for testosterone(see Sharpe, 1983 for review) and would also provide a mechanism for ensuring that such local differences in testosterone levels were maintained. The morphological data presented by Bergh(1982) shows that, in the rat, Leydig cells close to seminiferous tubules at stages VII-VIII are significantly larger than those close to other stages. This clearly suggests some stage-dependent differences in the secretion of certain factors which can affect the Leydig cells. Whilst studies by Syed et al(1985) suggest that only stages VII-VIII exert inhibitory effects upon unpurified cells this does not hold for the ability of tubules to stimulate Percoll purified cells since all stages exert a stimulatory

effect.

There was no effect of an LH antiserum given *in vivo* upon the subsequent stimulatory effects of medium conditioned by seminiferous tubules from these animals. This contrasts with findings that seminiferous tubule testosterone levels were reduced in these animals and that levels of the testicular interstitial fluid factor(Sharpe & Cooper, 1984) were elevated following injection of LH antiserum. These latter results suggested that some effect upon the ability of seminiferous tubules to alter testosterone production might be expected. The reason why this effect was not seen is not readily apparent. However, cryptorchidism and hypophysectomy both reduced the ability of seminiferous tubule-conditioned medium to stimulate Leydig cell testosterone production. Whilst these effects may be due to the reduced amounts of tissue added to condition these media, the numbers of Sertoli cells within the seminiferous tubules should have remained constant following these treatments and it is this aspect which is thought to be most important. Both cryptorchidism and hypophysectomy cause significant changes in Sertoli cell function(Jegou *et al*, 1983; Hansson *et al*, 1976; Ritzen *et al*, 1981), either directly, or via the disruption of hormonal supply and of Sertoli-germ cell interactions. In animals in which increasing degrees of testicular damage were induced by cryptorchidism and hypophysectomy, the ability of medium conditioned by seminiferous tubules from these testes to elicit a stimulatory response from the Leydig cells fell as the damage became more progressive . Thus, seminiferous tubule-conditioned medium from the most severely damaged testes, i.e. from the cryptorchid testes of hypophysectomised rats, had no stimulatory effect whatsoever upon Leydig cell testosterone

production. The ability of seminiferous tubule-conditioned medium from each of the treatment groups to enhance Leydig cell testosterone production varied inversely with the observed degree of testicular and tubular damage, as indicated by both testicular weight and testicular morphology(Kerr & Sharpe, 1985a). The results presented are consistent with the possibility that the Sertoli cell is the origin of the active factor(s) described in these experiments.

It could be argued that the effects of seminiferous tubule-conditioned media on Leydig cell function were aspecific, but, during this study, no effects of muscle conditioned medium were seen, suggesting that the effects seen are not aspecific tissue effects. Further evidence for the specificity of these results was provided by the results described above using tissue from cryptorchid, hypophysectomized and cryptorchid-hypophysectomized animals, which showed that using damaged seminiferous tubules the stimulatory effect could be negated.

Attempts to concentrate the factor(s) present in seminiferous tubule-conditioned medium were only partially sucessful and did not eliminate the variability of the results described above. This suggests that such variability is more likely to be due to variation in Leydig cell preparations or to the presence of more than one factor in the conditioned medium. Further studies upon the composition of the factor(s) in seminiferous tubule medium were hampered by the variability of the techniques used and the time required to prepare the large amounts of medium needed in these experiments.

The described system of incubation of Leydig cells in seminiferous tubule-conditioned medium provided a potential model for the <u>in vitro</u> study of the interaction between seminiferous tubules

and Leydig cells. However, due to both the lack of complete consistancy and the fact that the use of seminiferous tubule-conditioned medium allowed investigation only of one-way communication between these cell types the system is not ideal. The ideal system would be one in which the dynamic interactions between Leydig cells and seminiferous tubules could be investigated, as this at least approximates the physiological situation. Co-cultures of seminiferous tubules and Leydig cells would satisfy this requirement, but this system gave negative results, although other systems for the assessment of such interactions have been reported(Parvinen et al, 1984; Tabone et al, 1984). Static cultures also limit the assessment of the kinetics of hormone-cell interactions and tissue-tissue communications. Recently a column perifusion system has been validated for the investigation of Leydig cell desensitization by LH(Wu et al, 1985). Therefore as a logical extension of these studies on the interaction between seminiferous tubules and Leydig cells in static culture, studies using the perifusion system to investigate the dynamic interaction between seminiferous tubules and Leydig cells were undertaken and are described in Chapter 5.

CHAPTER 5

CO-PERIFUSION OF SEMINIFEROUS TUBULES AND LEYDIG CELLS

5.1: Introduction:

Static incubation systems do not provide an ideal method for the investigation of cellular function in vitro for a number of reasons: Firstly, the incubation medium is in a constant state of change due to cellular activity which produces substrate depletion, hormonal breakdown, accumulation of waste products and eventually exhaustion of the medium. Also with static systems the scope for the investigation of dynamic interactions between cell types or the dynamics of hormone action are limited by the volume of the culture which imposes limits on the frequency of sampling possible. Studies upon perfused testes(Chubb & Ewing, 1979a-c) have provided information on the biosynthetic pathways within the testis, but such studies do not provide detailed information on the interactions between individual components of the testis. The recent development and characterization of a perifusion system for Leydig cells, i.e. a system in which isolated cells are suspended in a gel matrix through which culture medium is passed, provided an opportunity to overcome these drawbacks(Wu et al, 1985). With this system, Wu and co-workers confirmed earlier observations by Davies & Platzer(1981) and Segaloff, Puett and Ascoli(1981) that repeated stimulation of Leydig cells by LH or hCG resulted in desensitization of Leydig cells when measured by the testosterone response to LH stimulation. Wu et al(1985) showed that the desensitization of Leydig cells was not due to cellular damage or to changes in the concentration of hCG/LH receptors. Furthermore such desensitization was dependent upon the dose of LH administered, with low doses (<0.01 ng/ml) producing a priming or sensitizing effect. Wu and co-workers therefore optimized and characterized the perifusion of

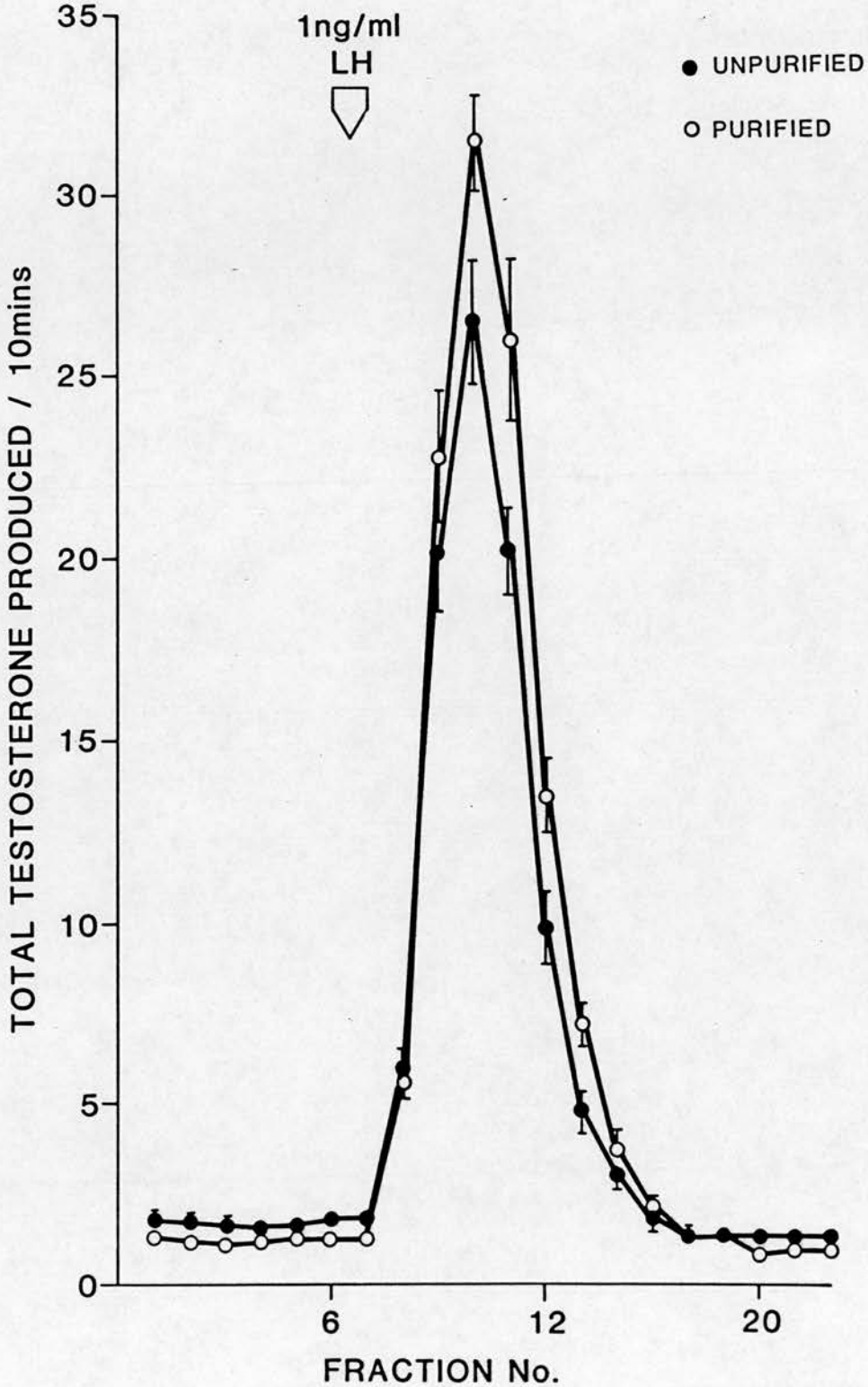
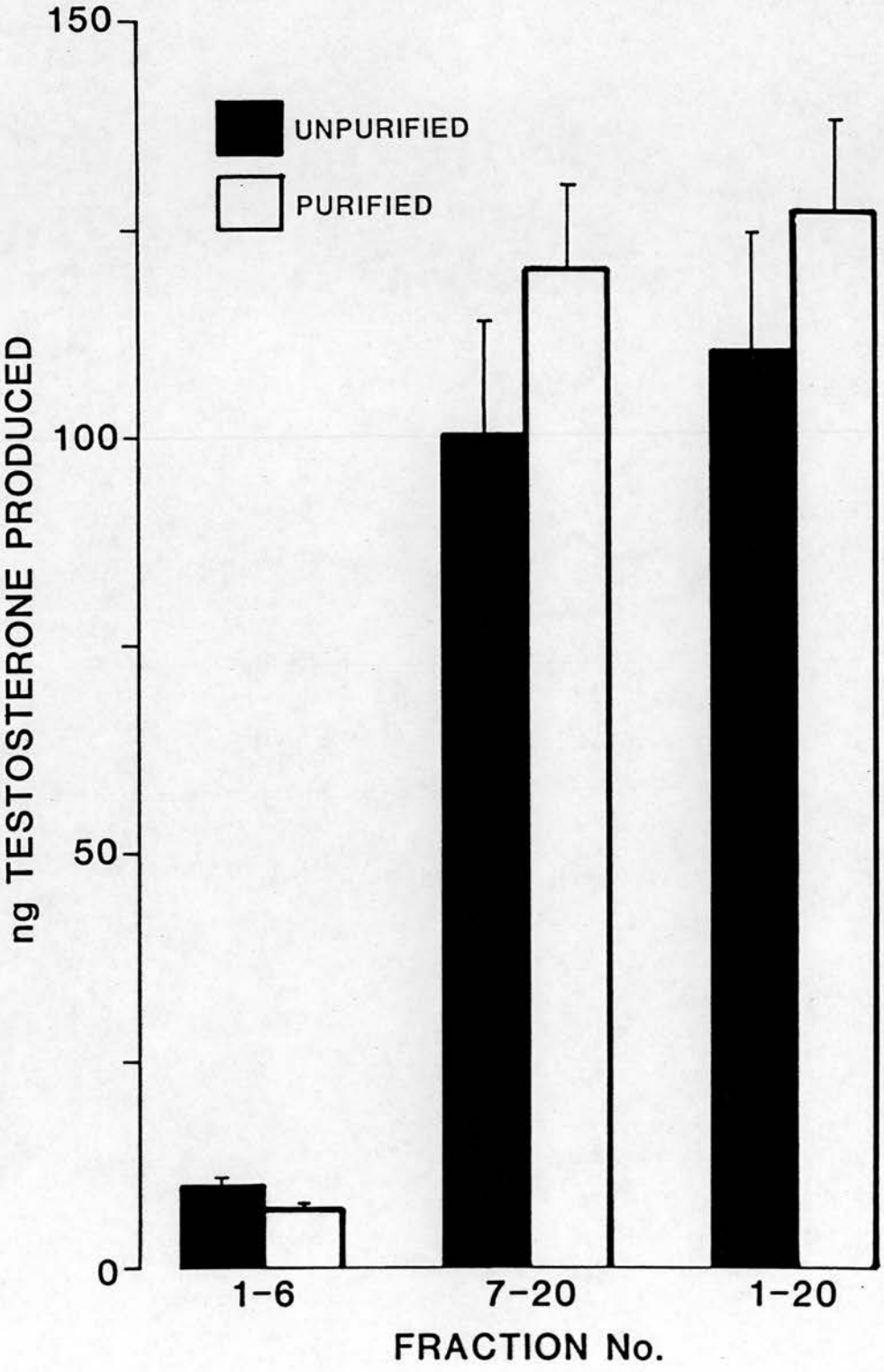


Fig 5.1: Comparision of the response to oLH stimulation of unpurified and Percoll purified Leydig cells during perifusion on Biogel columns. (Mean+/-s.d. of quadruplicate columns). Above: Profile of response to a single pulse of LH for 10 mins. Facing: Total testosterone produced before LH stimulation(Fractions 1-6), after LH stimulation(Fractions 7-20) and overall(Fractions 1-20).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Leydig cells such that a robust and reliable system was developed. As an extension to previous studies on static incubations for investigating interactions between seminiferous tubules and Leydig cells(see Chapters 3 & 4) the ability of seminiferous tubules to alter the steroidogenic capacity of Leydig cells was investigated using this perifusion system.

These studies involved collaboration with Dr. F.C.W. Wu and Dr. R.M. Sharpe for some parts of the preparation and running of columns.

5.2: Validation and testing of the perifusion system:

A number of experiments were carried out to establish the suitability of the system for studies in which seminiferous tubules and Leydig cells were co-perifused.

5.2.1: Comparison of the use of purified and unpurified Leydig cells:

The responsiveness of Percoll purified and unpurified cells in this system was determined. 4 columns were loaded with 1.7 million Percoll-purified Leydig cells and 4 others with 16.6 million unpurified cells. All columns were perifused(as described in Chapter 2) with medium alone for 60 min prior to a 10 minute 'pulse' of 1 ng oLH/ml being introduced. The response profiles of cells before and after Percoll purification were essentially identical(Fig 5.1), although the testosterone output per million cells was obviously lower for the unpurified cell preparation. Percoll-purified cells also showed a greater increase in testosterone production following oLH stimulation, showing a peak response of 25 x basal levels compared to a peak response of just 16 x basal levels for the unpurified cell preparation.

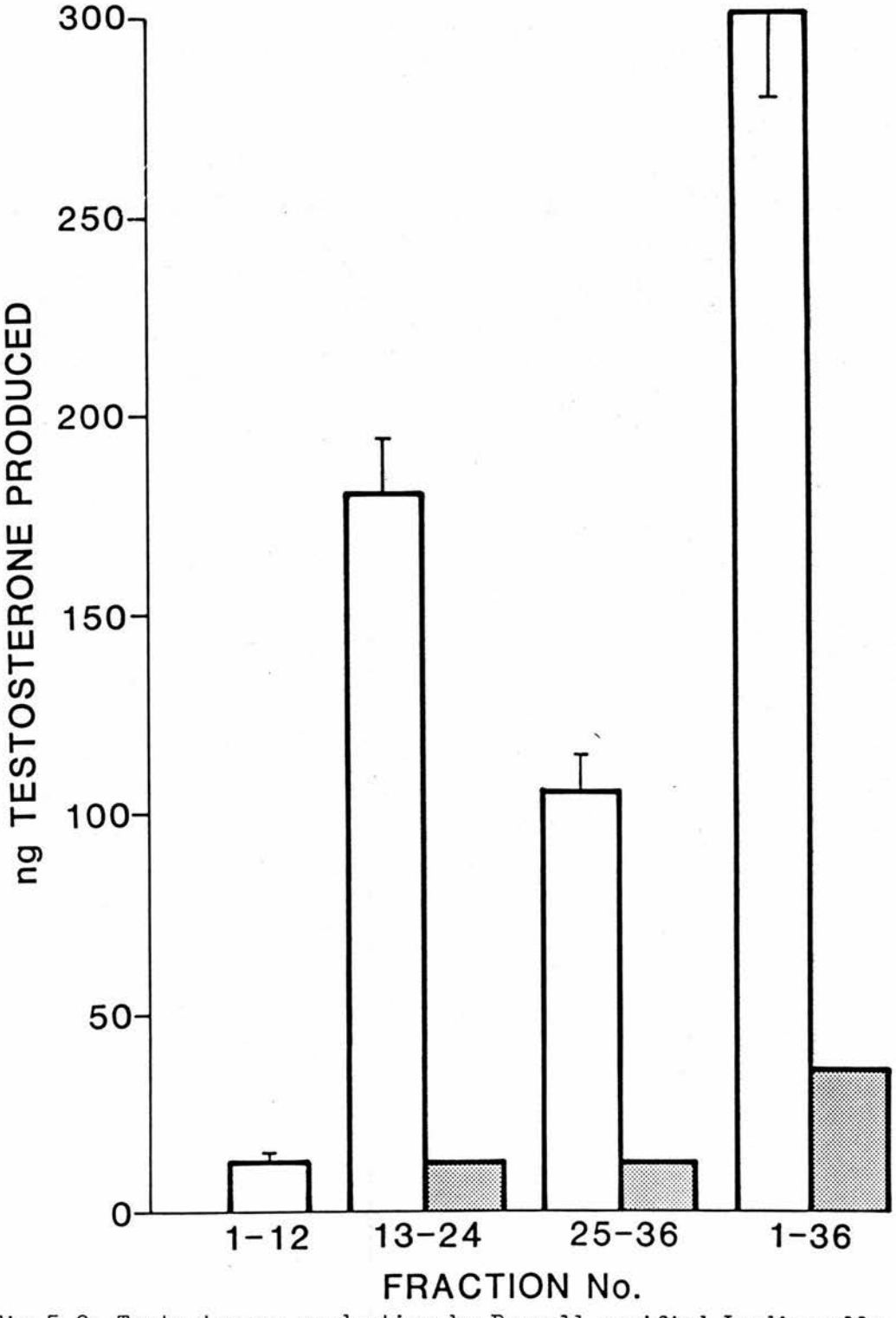


Fig 5.2: Testosterone production by Percoll purified Leydig cells on Biogel columns during 0-2h perifusion in the absence of LH stimulation (Fractions 1-12) and then from 2-4 h perifusion(Fractions 13-24) and from 4-6 h(Fractions 25-36) either with two 10 min pulses of LH at 2 & 4 hrs(open bars), or without LH stimulation(shaded bars). The total testosterone production during the entire perifusion period(Fractions 1-36) is also shown. Values are the means+/-s.d. of duplicate columns(open bars) or represent values from a single column perifused without LH stimulation(shaded bars).

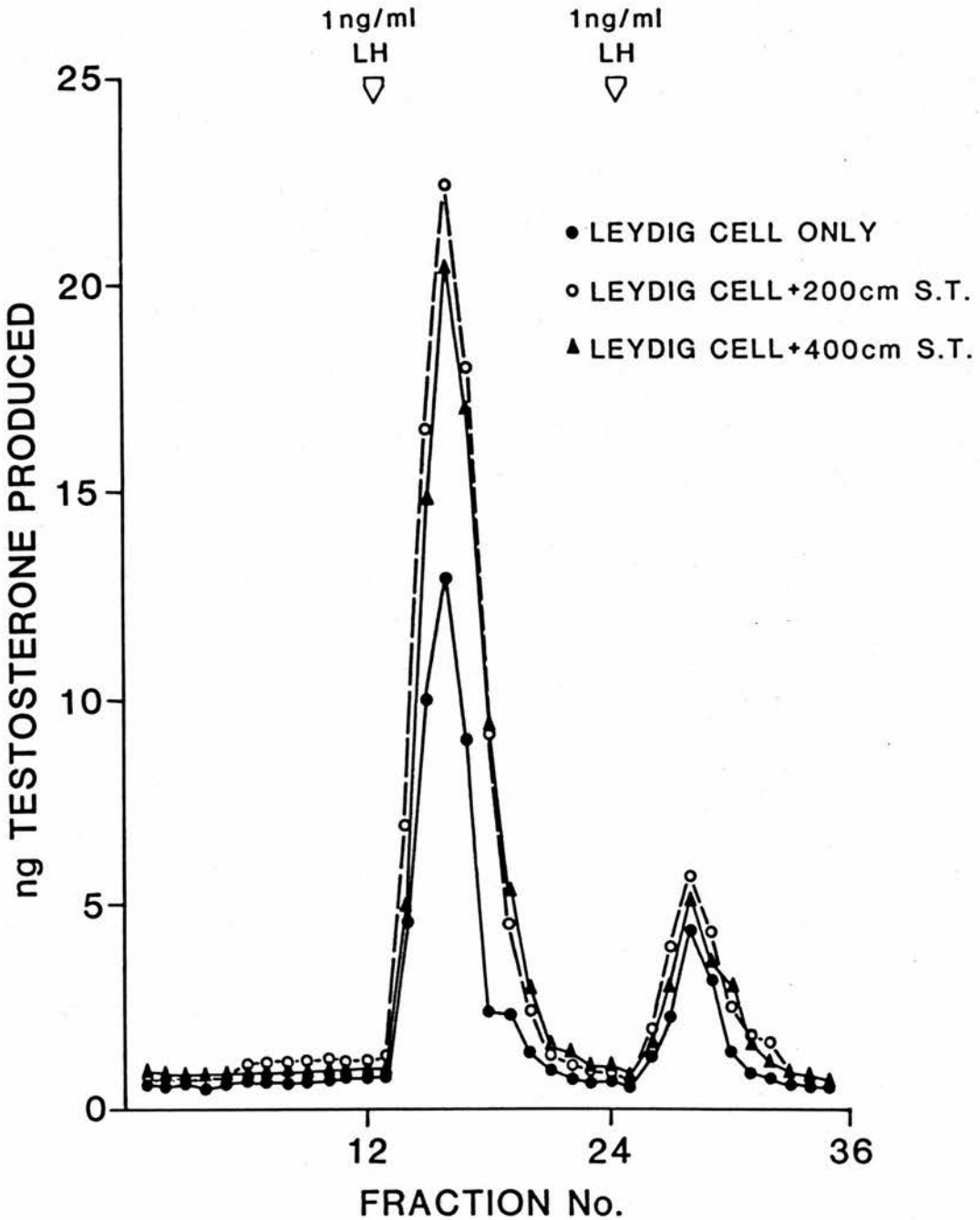
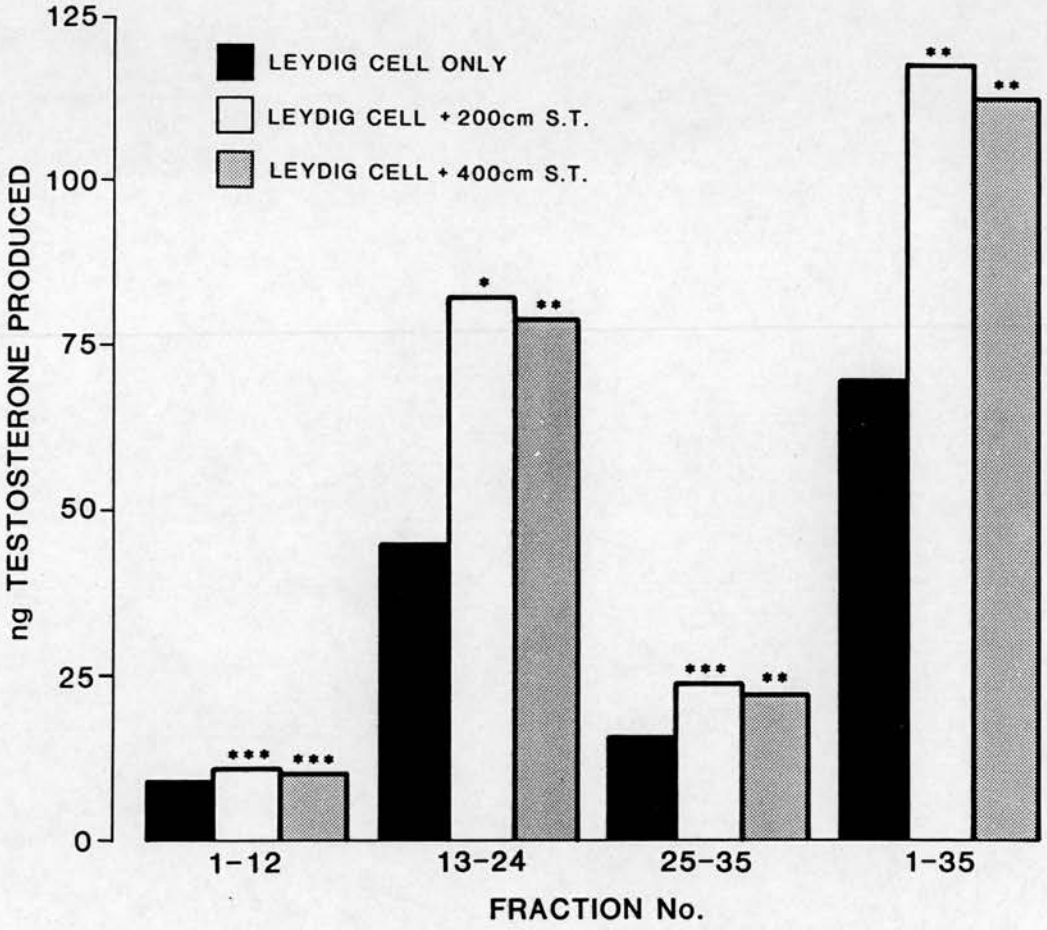


Fig 5.3: Effect of 200 or 400 cm of isolated seminiferous tubules on testosterone secretion by Percoll purified Leydig cells. Single columns were perifused for 2h in the absence of added oLH (Fractions 1-12), and then pulsed at 2 & 4 h with 1 ng/ml oLH. Total testosterone produced over 2h basally(Fractions 1-12) and during the 2h following the first(Fractions 13-24) and second(Fractions 25-35) LH pulses was calculated by summation of the testosterone produced for each of the 10 min fractions collected during these periods. Above: Profile of testosterone production/10 min fraction for each column. Facing: Total testosterone production over the first 2h (Fractions 1-12), and following the first(Fractions 13-24) and second (Fractions 25-35) LH stimuli, and for the duration of the experiment (Fractions 1-35). *Significant differences calculated by paired t-tests compared with testosterone secretion by Leydig cells alone (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).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5.2.2: Viability of Leydig cells during perifusion:

In a single experiment Percoll-purified cells perifused with and without the addition of oLH were compared. No difference in the basal testosterone output from Percoll-purified cells was observed over a 6 h period. i.e. there was no reduction in the ability of Leydig cells to produce testosterone during column perifusions between the first and sixth hour of perifusion(Fig 5.2). This data is in agreement with that of Wu et al,(1985) showing that no change in the ability of Leydig cells to respond to an initial stimulus of LH occurs during the first six hours of perifusion.

5.2.3: Optimization of the length of seminiferous tubules required for co-perifusion experiments:

In 3 separate experiments the ability of differing amounts of seminiferous tubules to alter Leydig cell testosterone production during co-perifusion was assessed. In 2 initial experiments single perifusion columns were loaded with either Leydig cells alone or with Leydig cells plus 200 or 400 cm(400 or 800 x 0.5 cm segments) seminiferous tubules. The results showed that while both 200 and 400 cm of seminiferous tubules enhanced the ability of Leydig cells to produce testosterone, 400 cm of seminiferous tubules enhanced Leydig cell testosterone production to a lesser degree than did 200 cm of seminiferous tubules(Fig 5.3). In a third experiment Percoll-purified Leydig cells were perifused alone or in the presence of 50, 100 or 200 cm seminiferous tubules. All columns were perifused for 2 h without added oLH stimulation, and then oLH was infused at 1 ng/ml for 20 min and subsequently for a further 10 min at 4 h. The results showed that the tubule concentration which maximized testosterone output by Leydig cells upon co-perifusion was 200 cm/column. Addition

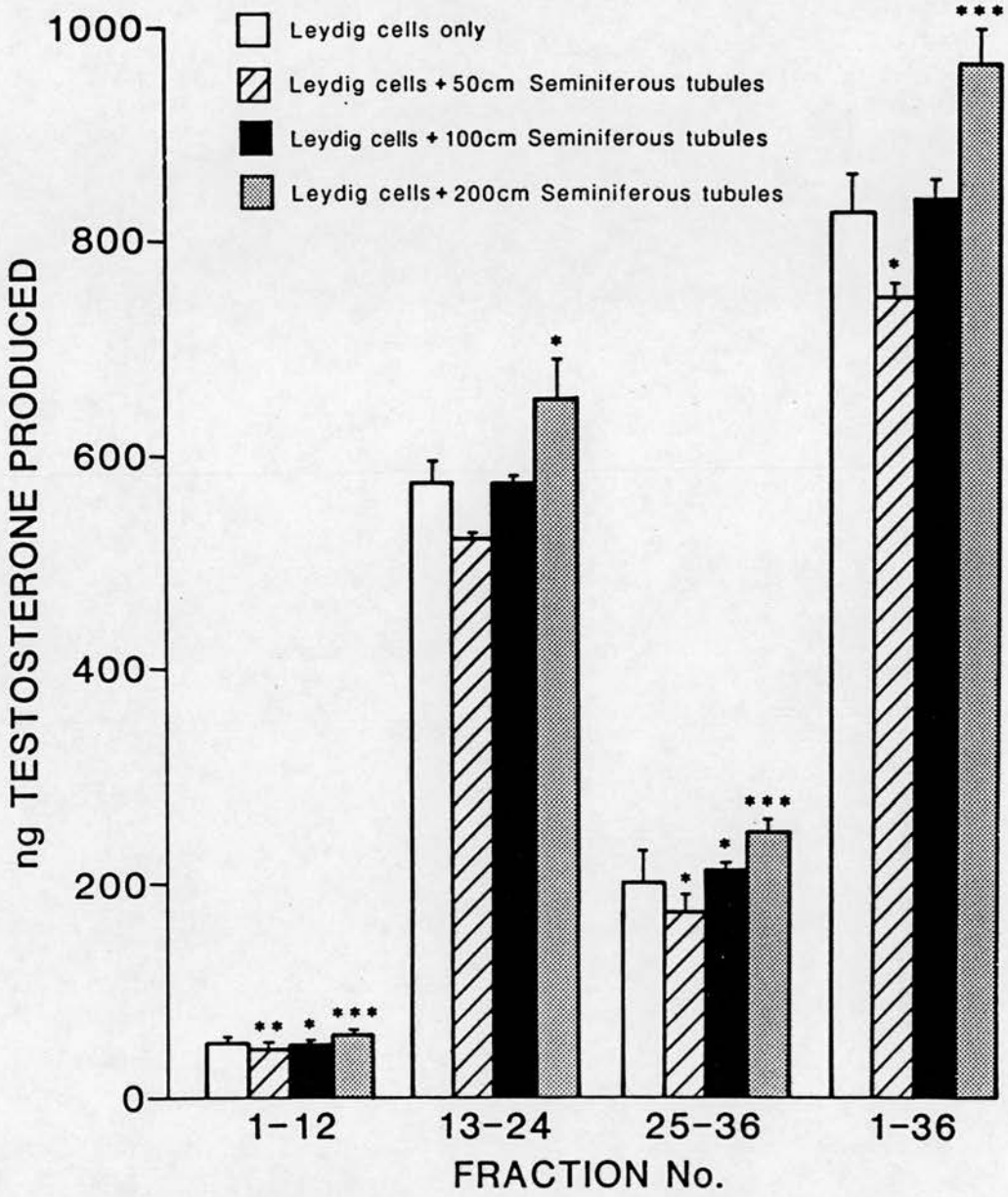


Fig 5.4: Effect of co-perifusion with 50, 100 cm or 200 cm of isolated seminiferous tubules on testosterone secretion by Percoll purified Leydig cells. Columns were perifused for 2h in the absence of LH(Fractions 1-12), and then pulsed at 2 & 4 h with 1 ng/ml oLH. Total testosterone produced over 2h basally(Fractions 1-12) and during the 2h following the first(Fractions 13-24) and second(Fractions 25-36) LH pulses was calculated by summation of the testosterone produced for each of the 10 min fractions collected during these periods(See Fig 5.1 for typical profile of the response of Leydig cells to pulsing with LH). Total testosterone produced throughout the experiment(i.e. over 6h) was calculated by the summation of all fractions(1-36) collected for each column. Results are shown as the Mean+/-s.d. of testosterone produced during 2h or 6h for 2 columns. *Significant differences compared with testosterone secretion by Leydig cells alone (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

of 50 cm/column of seminiferous tubules resulted in a significant decrease in Leydig cell testosterone output during the initial 2 h basal perifusion phase($p<0.01$ vs controls) and also during the second oLH stimulation(4-6 h, $p<0.02$) and overall($p<0.02$, Fig 5.4). With 100 cm/column there was little difference in testosterone output between control columns and those containing seminiferous tubules, although slight but significant increases were seen during the initial basal perifusion(0-2 h, $p<0.02$) and during the second oLH peak(4-6 h, $p<0.05$). However no significant change in testosterone output was seen overall when Leydig cells were co-perifused with 100 cm of seminiferous tubules(Fig 5.4). Columns containing 200 cm of seminiferous tubules plus Leydig cells produced significantly more testosterone than did controls containing Leydig cells alone, both during a basal 2 hour period(12% increase over controls, $p<0.001$) and during the first(14% increase, $p<0.02$) and second(24% increase, $p<0.001$) oLH 'pulses'(Fig 5.4). The overall increase in testosterone production with 200 cm seminiferous tubules per column for the total perifusion period was in excess of 130 ng testosterone per column ($p<0.001$, Fig 5.4).

5.3: Perifusion of Leydig cells and seminiferous tubules alone or in combination:

In 2 separate experiments, it was established that the increase in testosterone output by Leydig cells co-perifused with seminiferous tubules could not be attributed to leakage of testosterone from the seminiferous tubules. In both experiments, 6 columns were perifused; 2 containing Leydig cells alone, 2 containing both Leydig cells and seminiferous tubules and 2 containing equivalent amounts of seminiferous tubules but no Leydig cells. In both

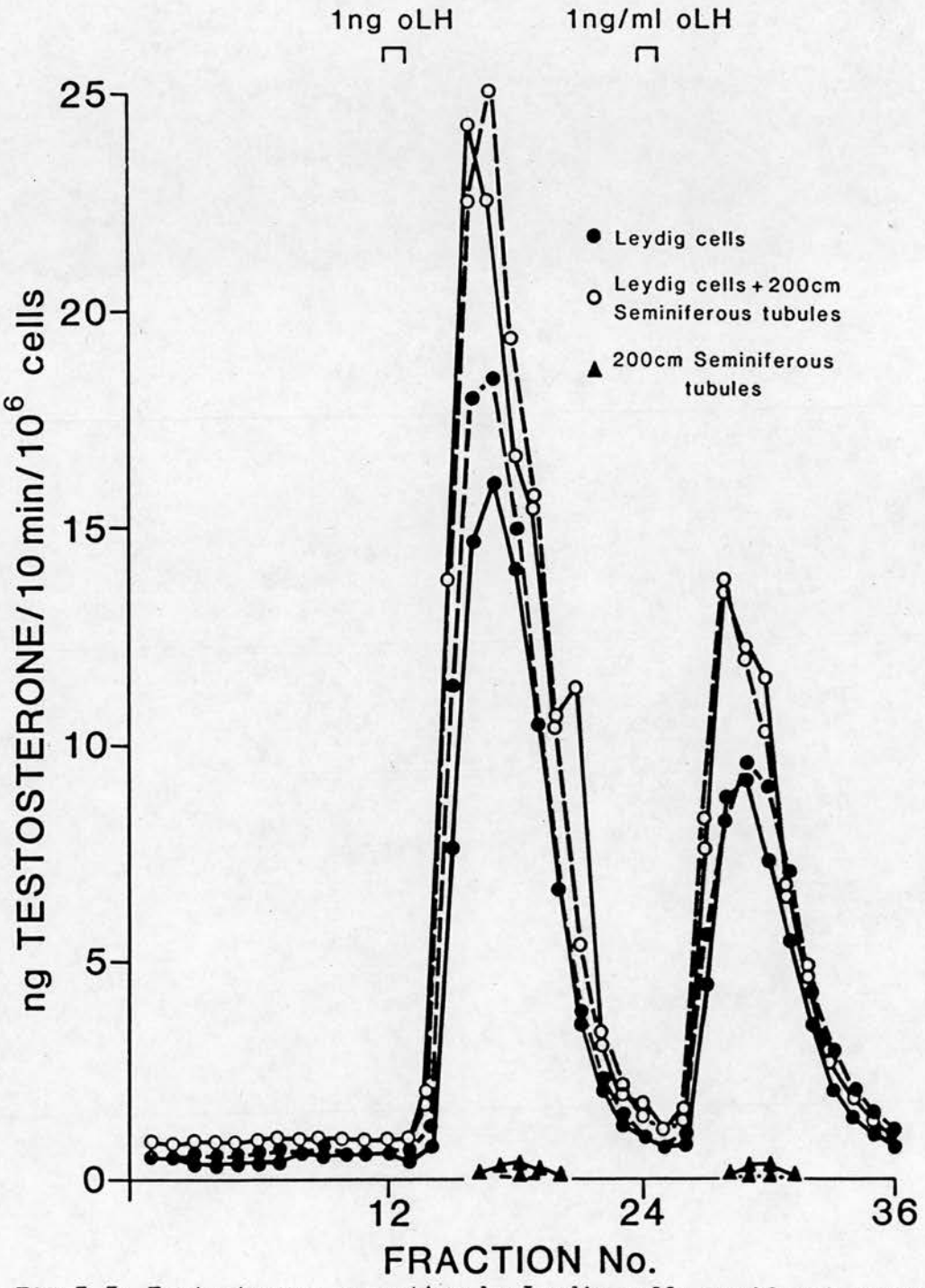


Fig 5.5: Testosterone secretion by Leydig cells perifused alone or co-perifused with 200 cm seminiferous tubules. Columns were perifused for 2h in the absence of added LH and then 'pulsed' at 2 & 4h with 1ng/ml oLH for 10 min. Testosterone secretion from seminiferous tubules perifused alone was undetectable at a flow rate of 0.5mls/min(not shown) and was only just detectable during LH stimulation at a flow rate of 0.25ms/min(see Fig).

experiments no detectable testosterone was measured in the perifusate from either of the 2 columns containing seminiferous tubules only in the absence of oLH. In one experiment, following oLH stimulation, small amounts(<200 pg in total) of testosterone were measured in the perifusate. However this amount, when compared with the 16 ng testosterone/million cells for the Leydig cell controls and the 25 ng testosterone/million cells for the co-perifusion columns was able to account for only a tiny fraction of the 9 ng/million cells difference between these treatments(Fig 5.5). The most likely explanation for the presence of testosterone in the effluate of the seminiferous tubule only columns is that during the dissection procedure small numbers of Leydig cells had been carried over. By comparison with the columns in which Leydig cells and seminiferous tubules were co-perifused, it could be seen that the testosterone produced by columns containing seminiferous tubules alone represented less than 8,000 Leydig cells. It should be noted that if cells were carried over, these experiments also prove that such contamination would be far too small to explain the effects of seminiferous tubules upon Leydig cell testosterone output.

However as an additional control procedure, using both teased and collagenase-digested seminiferous tubules from rats aged 35, 55 and 70 days, hCG binding assays were carried out upon both seminiferous tubules and Leydig cells. The results showed less than 0.5% specific binding of hCG to isolated seminiferous tubules(See Chapter 6 below), and this binding was never shown to be significantly different from non-specific binding values, suggesting that few, if any, contaminating Leydig cells were present. Leydig cells, used as a control, showed significant($p<0.001$, Students t-test) specific binding

COL ADDS	oLH DOSE (ng/ml)	FRACTION NUMBER 1-12	13-24	25-36	1-36
LC	0.01	6.0+/-0.7	9.2+/-2.1	11.3+/-0.6	26.5+/-3.4
LC+ST	0.01	7.7+/-0.2 p<0.001	9.3+/-2.0	10.4+/-2.5 p<0.001	27.4+/-4.4
LC	0.1	7.8+/-0.1	13.0+/-0.3	13.8+/-1.1	34.5+/-1.4
LC+ST	0.1	10.3+/-1.5 p<0.001	18.8+/-2.3 p<0.001	18.3+/-2.2 p<0.001	47.4+/-5.9 p<0.001
LC	1.0	7.5+/-0.4	78.6+/-7.5	47.6+/-0.5	133.7+/-8.4
LC+ST	1.0	10.1+/-1.4 p<0.001	137.6+/-5.3 p<0.01	78.5+/-1.5 p<0.001	226.2+/-5.2 p<0.001
LC	10.0	6.4+/-1.1	139.6+/-21.7	71.8+/-2.6	217.9+/-25.4
LC+ST	10.0	9.2+/-0.1 p<0.001	176.6+/-10.9 p<0.001	92.1+/-4.3 p<0.001	277.9+/-15.3 p<0.05

Table 5.1: Effect of varying oLH stimulation on testosterone production(ng/10^6cells) by Leydig cells(LC) on their own or co-perifused with seminiferous tubules(LC+ST). The mean(+/- s.d.) response of duplicate columns are shown over the 2 h period preceding the first LH stimulus(Fractions 1-12) and following the first (Fractions 13-24) and second LH stimuli(Fractions 25-36), and also for the experiment as a whole(Fractions 1-36). 'p' values shown indicate significant differences compared with testosterone secretion by Leydig cells alone.

of hCG. In these experiments(Fig 5.5) testosterone production by Leydig cells co-perifused with seminiferous tubules was significantly higher than by Leydig cells alone both during the initial basal phase ($p<0.001$ for both experiments), and following the first($p<0.01$ & 0.001 respectively) and second($p<0.001$ & 0.02 respectively) oLH stimuli as well as during the whole perifusion period($p<0.001$ for both experiments).

5.4: Effect of varying oLH stimulation on the response of Leydig cells co-perifused with seminiferous tubules:

In a series of co-perifusion experiments the effect of varying the degree of oLH stimulation was determined. Using a range of ovine LH concentrations from 0.01-10ng/ml duplicate columns containing both seminiferous tubules plus Leydig cells and also duplicate control columns containing Leydig cells only were prepared. Four levels of oLH stimulation were assessed, 0.01, 0.1, 1.0 and 10.0 ng/ml. In all experiments under basal conditions the presence of seminiferous tubules enhanced Leydig cell testosterone production when compared with Leydig cell-only control columns(Table 5.1).

Leydig cells alone, responded to increasing doses of oLH by producing progressively more testosterone. Thus, in the absence of LH stimulation, testosterone production over 2 h was about 6.9ng/10^6cells, increasing to 9.2ng/10^6cells in the presence of 0.01ng/ml oLH, 13.0ng/10^6cells for 0.1ng/ml oLH, 78.6ng/10^6cells for 1.0ng/ml oLH and reaching 139.6ng/10^6cells in the presence of 10.0ng/ml oLH. In contrast to this almost linear dose-response of Leydig cells alone to oLH stimulation, the effect of seminiferous tubules upon Leydig cell responsiveness followed a markedly different pattern. During perifusion for 2 h in the absence of oLH stimulation,

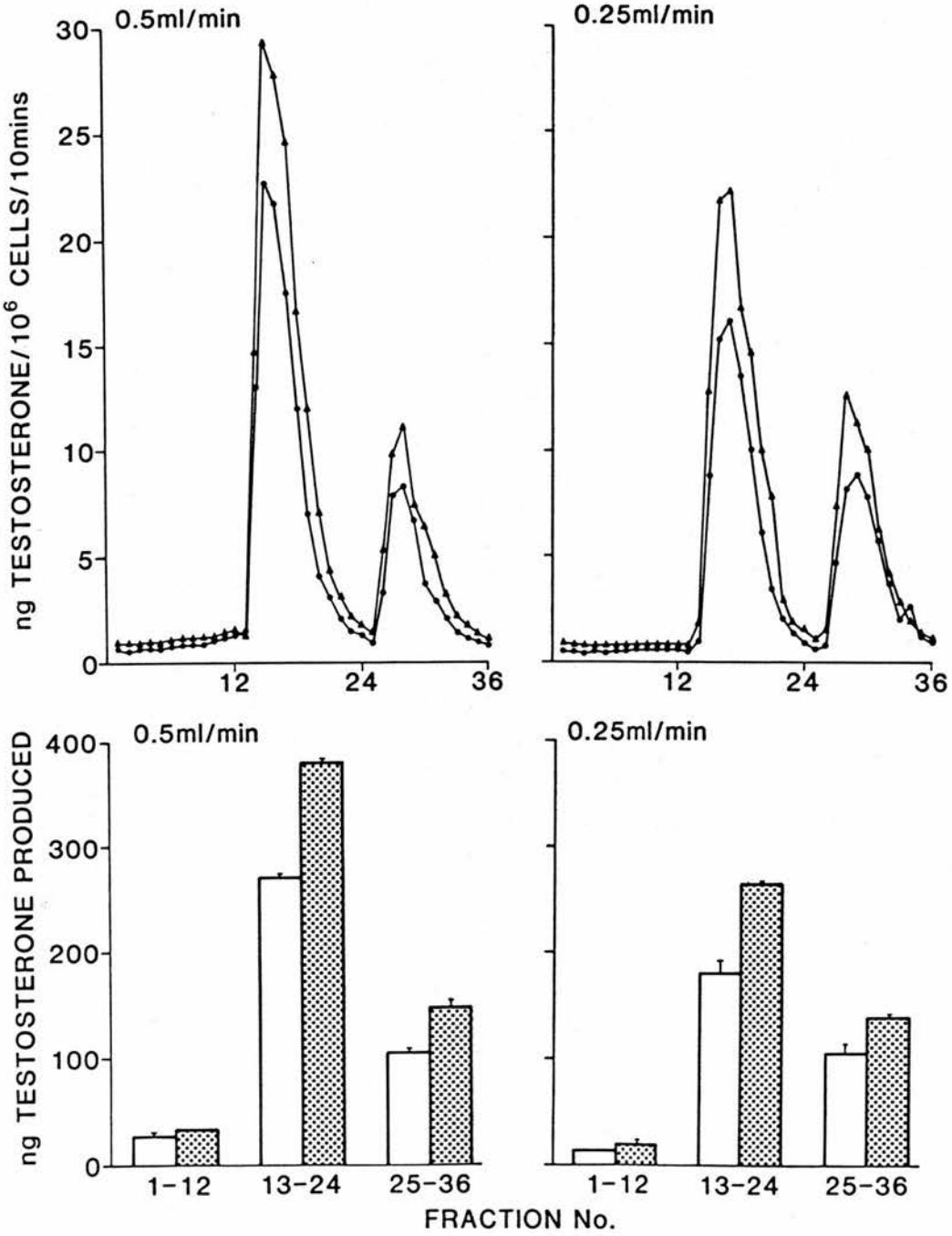
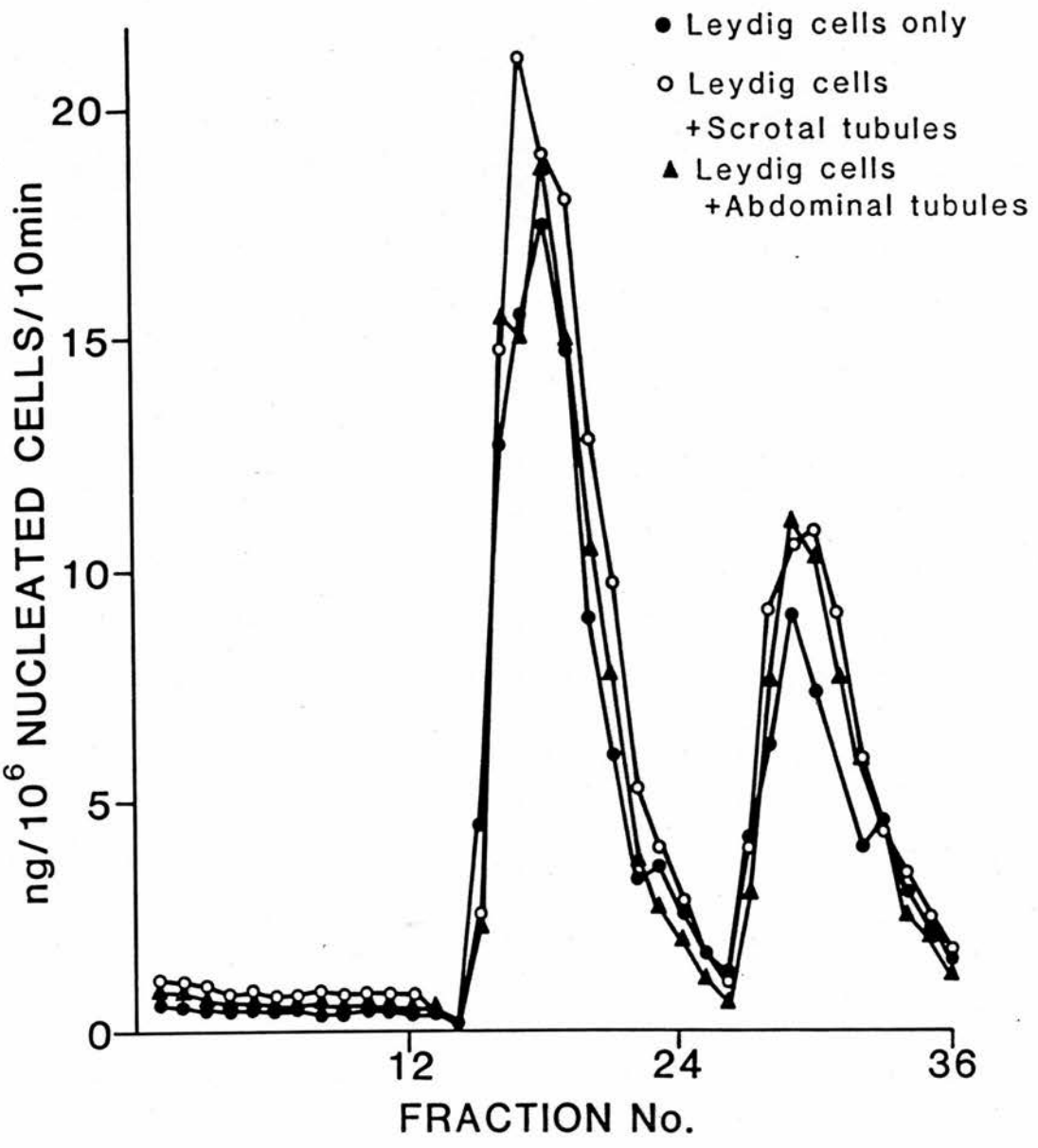


Fig 5.6: Effect of altering the flow rate on the perifusion system: Columns were perifused in two separate experiments, at either 0.5 ml/min(Left panels) or at 0.25 ml/min(right panels). The stimulatory effect of isolated seminiferous tubules on testosterone secretion by Percoll purifed Leydig cells persists basally(Fractions 1-12) and following two separate pulses of 1 ng/ml oLH(Fractions 13-24 & 25-36) at both flow rates. Profiles(top panels) and total testosterone production/2h(bottom panels) are from duplicate columns).

seminiferous tubules significantly increased Leydig cell testosterone production by 2.4ng/10^6cells/2h(34+/-2.5%, mean+/-sem, increase over controls; $p<0.001$ in all 4 experiments when compared with controls). Following stimulation with 0.01 ng/ml oLH this effect of the tubules was lost, there being no significant increase in Leydig cell testosterone production induced by seminiferous tubules when compared with Leydig cells alone during the first LH stimulus(2-4 h), and a slight but significant decrease during the second LH stimulus(4-6 h, $p<0.001$). Thereafter, at increasing LH doses, the effect of seminiferous tubules upon the Leydig cells, over and above that of LH, increased at 0.1 & 1.0 ng/ml oLH(by 58 & 59 ng/10^6cells/2h, 46% & 75%, $p<0.001$ & 0.001 above controls respectively) but this increment was lower(37ng/10^6cells/2h, 26%, $p<0.001$ above controls) when a maximally -stimulating dose of oLH, i.e. 10.0 ng/ml, was infused(Table 5.1). This pattern was repeated following the second LH stimulus and overall throughout the perifusion period(Table 5.1). Overall only with the 0.01 ng/ml LH dose did seminiferous tubules fail to produce a stimulatory effect.

5.5: Effect of altering the flow rate of medium on the perifusion system:

In 2 separate experiments the effect of altering the flow-rate of medium through the perifusion system was determined. Duplicate columns containing seminiferous tubules plus Leydig cells or Leydig cells alone were perifused at flow rates of 0.5 and 0.25mls/min. Whilst altering the flow rate had little effect on the degree of enhancement caused by seminiferous tubules of oLH stimulated testosterone production by Leydig cells, during the initial basal period seminiferous tubules co-perifused with Leydig cells at 0.25ml/min showed a 75% increase over controls while those co-perifused at


● Leydig cells only
o Leydig cells
+Scrotal tubules
▲ Leydig cells
+Abdominal tubules
ng/10^6 NUCLEATED CELLS/10min
0
5
10
15
20
12
24
36
FRACTION No.

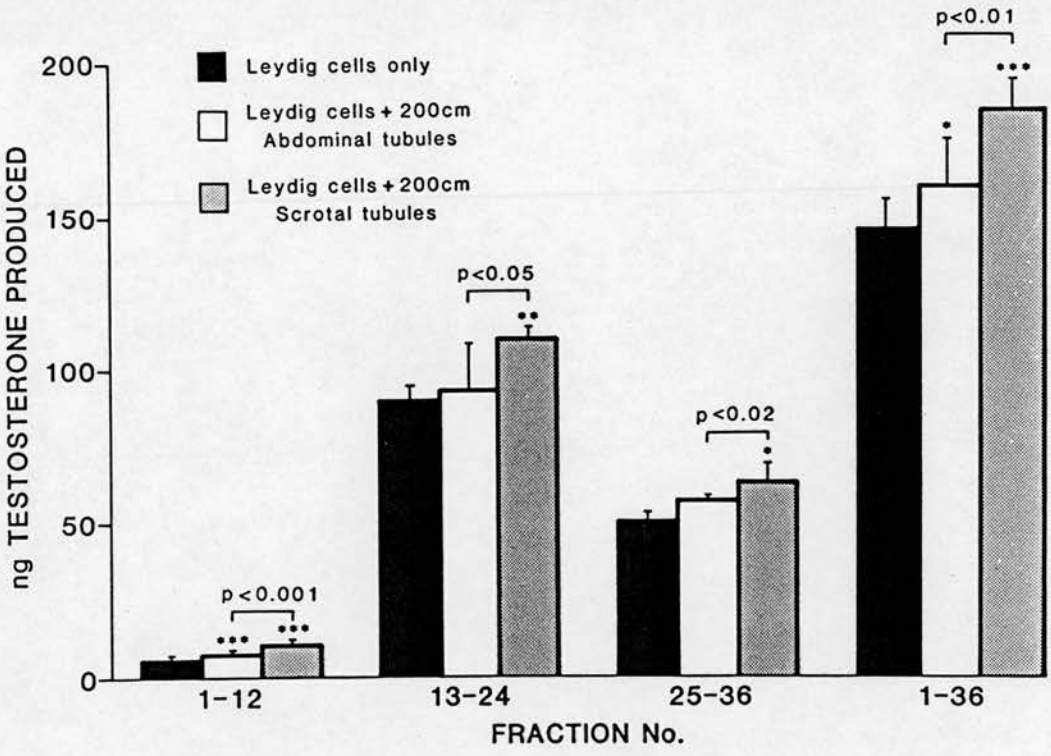


Fig 5.7: Testosterone secretion by Leydig cells perifused alone, or co-perifused with isolated seminiferous tubules from either the abdominal or scrotal testes of rats made unilaterally cryptorchid six days prior to death. Tubules from scrotal testes significantly increased Leydig cell testosterone secretion basally over 2 h (Fractions 1-12) and also following the first(Fractions 13-24) and second LH pulses(Fractions 25-36) and over the whole experiment (Fractions 1-36), when compared either with control Leydig cells alone or with Leydig cells co-perifused with tubules from abdominal testes. *Significant differences compared with testosterone production by Leydig cells alone(* $p<0.05$; ** $p<0.02$; *** $p<0.001$). Facing: Profile of response to LH stimuli(Mean of two columns in each case). Above: Total testosterone produced over the first 2 h of perifusion (Fractions 1-12) and in the 2 h following the first(Fractions 13-24) and second 25-36) LH stimuli, as well as the total testosterone production over 6 h(Fractions 1-36). Mean+/-s.d. of duplicate columns.

0.5 ml/min showed only a 33% increase over control values(Fig 5.6). Again the testosterone production by Leydig cells co-perifused with seminiferous tubules was significantly higher than control columns containing Leydig cells only at all points throughout the perifusion period(Table 5.2).

5.6: Effect of co-perifusion of Leydig cells with seminiferous tubules from normal and cryptorchid testes:

Rats were made unilaterally cryptorchid six days prior to death, and seminiferous tubules of approximately equivalent tissue mass were prepared from the abdominal and scrotal testes. Short term cryptorchidism provided a model for the investigation of impaired Sertoli cell function without gross changes in the wet weight of the abdominal and scrotal testes or seminiferous tubules from these testes (See Sharpe et al, 1984). Duplicate columns were prepared containing 2 million Leydig cells plus or minus 200 cm of isolated tubules from either cryptorchid(abdominal) or normal(scrotal) testes. Columns were perifused for 6 h at a flow rate of 0.25 ml/min. During the initial basal phase of the perifusion, Leydig cells perifused in the presence of seminiferous tubules from abdominal testes produced significantly less testosterone than did Leydig cells co-perifused with seminiferous tubules from scrotal testes($p<0.001$, Fig 5.7). Seminiferous tubules in both cases produced significant increases(abdominal 33%, scrotal 77%, $p<0.001$) in Leydig cell testosterone production during the initial basal phase(Fig 5.7). During the first 'pulsing' of the system with oLH, seminiferous tubules from the scrotal testes significantly enhanced Leydig cell testosterone production when compared both with columns containing Leydig cells alone(23%, $p<0.02$) and columns in which tubules from cryptorchid testes were co-perifused

EXPT No.	COLUMN	FRACTION NUMBER 1-12	13-24	25-36	1-36
1	LC	9.4	47.4	15.1	71.9
	LC+ST	11.8 p<0.001	88.6 p<0.001	20.5 p<0.001	120.9 p<0.01
2	LC	16.6+/-0.9	182.4+/-6.2	64.1+/-2.8	263.1+/-9.9
	LC+ST	18.5+/-0.5 p<0.001	208.1+/-7.3 p<0.02	79.3+/-2.7 p<0.001	305.6+/-9.9 p<0.001
3	LC	10.4+/-1.5	102.7+/-1.9	39.7+/-1.5	152.7+/-4.9
	LC+ST	13.0+/-0.1 p<0.001	144.6+/-4.1 p<0.01	55.8+/-1.9 p<0.001	213.3+/-6.2 p<0.001
4	LC	7.2+/-0.3	92.9+/-6.7	54.4+/-4.2	154.4+/-11.2
	LC+ST	10.6+/-0.6 p<0.001	134.9+/-1.2 p<0.001	71.6+/-0.9 p<0.02	217.1+/-2.8 p<0.001
5	LC	7.5+/-0.4	78.8+/-7.7	47.6+/-0.5	133.7+/-8.4
	LC+ST	10.1+/-1.4 p<0.001	137.7+/-5.3 p<0.01	78.5+/-1.6 p<0.01	226.2+/-5.2 p<0.001
6	LC	5.9+/-0.5	89.7+/-6.5	49.1+/-0.5	146.1+/-9.3
	LC+ST	10.5+/-1.3 p<0.001	110.5+/-3.6 p<0.02	63.6+/-5.6 p<0.05	184.7+/-10.0 p<0.001
1-6	LC	9.5+/-3.8	99.0+/-45.1	45.0+/-16.7	153.7+/-61.8
	LC+ST	12.4+/-3.2 p<0.001	137.4+/-40.4 p<0.01	61.6+/-22.0 p<0.01	211.3+/-60.1 p<0.001

Table 5.2: Reproducibility over 6 experiments of the stimulatory effects of seminiferous tubules on Leydig cell testosterone secretion (ng/10 cells) during co-perifusion. The mean(+/- s.d.) response of duplicate columns containing Leydig cells alone or Leydig cells co-perifused with seminiferous tubules are shown over the 2 h periods preceding the first LH stimulus(Fractions 1-12) and following the first(Fractions 13-24) and second(Fractions 25-36) stimuli with 1ng/ml oLH as well as results for the whole experiment(Fractions 1-36). 'p' values indicate significant differences compared with testosterone secretion by Leydig cells alone, analysed by paired t-tests between samples for individual experiments. Mean testosterone values per 2 h period and over the whole 6 h perifusion for six experiments are shown in the lower panel, and for this section significant differences were calculated using paired t-tests on the mean testosterone produced during the first 2 h(Fractions 1-12) and the 2 h following the first (Fractions 13-24) and second(Fractions 25-36) LH pulses and also during the whole experiment(Fractions 1-36).

with Leydig cells($p<0.05$). No significant effect of tubules from the abdominal testes on Leydig cell testosterone production was evident. During the second 'pulsing' of the system with oLH, seminiferous tubules from scrotal testes significantly enhanced Leydig cell testosterone production(26% over controls, $p<0.05$; 13% over abdominal tubules, $p<0.02$ Fig 5.7). No significant effect of tubules from abdominal testes was evident. Overall, whilst tubules from abdominal testes enhanced Leydig cell testosterone production significantly(10%, $p<0.05$ vs controls), tubules from normal, i.e. scrotal testes produced significantly greater enhancement of testosterone production throughout the total perifusion period(26%, $p<0.001$ vs controls, $p<0.01$ vs abdominal tubules, Fig 5.7).

5.7: Reproducibilty of results:

To assess the reliability and reproducibility of results obtained in this system the results from 6 experiments in which columns containing either Leydig cells alone or Leydig cells plus 200 cm seminiferous tubules, were perifused for up to 6 h were compared. Of these experiments, 5 employed duplicate columns per treatment whilst one employed only single columns. In all six experiments Leydig cells perifused in the presence of seminiferous tubules produced significantly greater amounts of testosterone than did Leydig cells perifused alone($p<0.05$-$p<0.001$, Table 5.2). During perifusion without added oLH, i.e. basally, Leydig cells co-perifused with seminiferous tubules produced an average of 27+/-5%(mean+/-sem, $p<0.01$-$p<0.001$) more testosterone than did Leydig cells perifused alone. During the 2 h following the first oLH stimulus the increment in testosterone production during co-perifusion of tubules with Leydig cells was 48+/-11%($p<0.02$-$p<0.001$), whilst during the period

following the second stimulus the increment was 37+/-6%($p<0.05$-$p<0.001$). Overall, during the whole 6 h of perifusion the presence of seminiferous tubules enhanced Leydig cell testosterone by an average of 43+/-8%($p<0.01$-$p<0.001$). This represents an average increase of 116 ng testosterone production. Over all six experiments it can be seen(Table 5.2) that the presence of seminiferous tubules produces a highly significant($p<0.01$-$p<0.001$) increase in Leydig cell testosterone production throughout all phases of the perifusion.

5.8: Discussion:

The development of a system for the investigation of dynamic interactions between the two major components of the testis, the seminiferous tubules and interstitial Leydig cells provides a tool which should enable a greater understanding of the subtle interactions between these compartments to be formed.

The system described allows the investigation of interactions between seminiferous tubules and unpurified or purified Leydig cells. These studies have concentrated upon the interactions between seminiferous tubules and Percoll-purified Leydig cells primarily because earlier studies suggested that this would be the most profitable approach(See Chapter 4). The results presented above have shown that whilst seminiferous tubule-conditioned medium stimulated testosterone production by Percoll-purified Leydig cells, no effect of such conditioned medium was observed upon unpurified interstitial cells(Chapter 4.2). The contrasting results obtained in other systems using non-purified and purified Leydig cells(Sayed et al, 1985, Parvinen et al, 1984, Ch 4) mitigated against the further study of non-purified cells in this system. However in the light of the results presented here it would appear likely that the perifusion

system will prove extremely useful for further studies comparing the dynamic effects of seminiferous tubules on purified and non-purified Leydig cells, and also that this area of study should prove both interesting and profitable.

The perifusion system has been previously validated for the study of non-purified Leydig cells(Wu et al, 1985), and these and the present studies have shown that Leydig cell responsiveness, and presumably therefore their viability, remains unchanged for at least 6h during basal perifusion.

In this system Leydig cells have been shown to respond in a dose-related manner to the presence of seminiferous tubules by increasing their output of testosterone. Both inhibitory and stimulatory effects have been observed, such effects being dependent upon the ratio of seminiferous tubules to Leydig cells within the columns. These results opened up two possible avenues for further study using the perifusion system; to investigate either the inhibitory or the stimulatory effects of seminiferous tubules upon Leydig cell testosterone production. Since the testicular content of both Leydig cells and seminiferous tubules are documented it was possible to assess the physiological ratio between these two tissues. On a per gram basis the adult rat testis contains approximately 19 million Leydig cells(Christensen & Peacock, 1980) and 1240 cm of seminiferous tubules(Wing & Christensen, 1982), or about 3.0 million Leydig cells/200 cm of tubules. The cell numbers selected for these experiments in the light of this observation were of the order of 1.5-3.0 million cells per 200 cm of tubules. In determining the optimum conditions for investigation of seminiferous tubule-Leydig cell interactions it is of interest that at a ratio of 3.15 million

cells/200 cm seminiferous tubules the maximum stimulatory effects upon Leydig cell testosterone production were seen, whilst at a ratio of 3.15 million cells to 50 cm seminiferous tubules an inhibitory effect of the tubules upon Leydig cell testosterone production was observed. In the light of these observations, initial investigations into the observed effects were centred upon the stimulatory effects of seminiferous tubules upon Leydig cells, since these occurred with "physiological" proportions of seminiferous tubules and Leydig cells. Further studies into the possible inhibitory role of seminiferous tubules could yield valuable information about possible inhibitory factors released by seminiferous tubules which act upon Leydig cells. That such inhibitory effects are not the result of the release of cellular toxins is borne out by the ability of concentrations of seminiferous tubules 4-8 times higher than those producing inhibitory effects to enhance Leydig cell testosterone production. The physiological significance of the stimulatory and inhibitory effects of seminiferous tubules to is not clear. Further studies will be required before any definite conclusions can be drawn concerning the precise nature of such inhibitory effects.

The described stimulatory effects of seminiferous tubules upon Leydig cell steroidogenesis have been studied under a wide range of conditions. Significant stimulation of Leydig cell testosterone production by seminiferous tubules has been described in 6 separate experiments when 1.0 ng/ml oLH was used as a stimulus, and in further experiments in which different doses of oLH were used. The effect persisted following cryptorchidism, although the magnitude of the stimulatory effect of the tubules on Leydig cell testosterone production was reduced. The effect also persisted

following alteration of the flow rate of medium through the system.

Overall, it would appear that the stimulatory effects of seminiferous tubules upon Leydig cell testosterone production are robust and reproducible. However, since no tissue exists which is comparable to the seminiferous tubules in both its structure and its complexity it was not possible to introduce a tissue control into the system. Therefore it may be argued that the observed effects are not tissue specific. There are two observations with which this argument may be answered: Firstly, in experiments in which Leydig cells were co-perifused with seminiferous tubules from the abdominal and scrotal testes of short-term unilaterally cryptorchid rats, the effect of the abdominal(cryptorchid) tubules upon Leydig cell steroidogenesis was markedly less than the effect of the scrotal(normal) tubules. This suggests that even minor disruption of the seminiferous tubules markedly affects their ability to stimulate Leydig cells. Secondly, in experiments in which the dose responsiveness of the co-perifused tissues to oLH was assessed, the degree of stimulation of Leydig cells by the seminiferous tubules was markedly affected by the presence of differing oLH doses. For example, in the absence of oLH, Leydig cell testosterone production was increased by 34% in the presence of seminiferous tubules, whilst in the same experiment the infusion of 0.01 ng/ml oLH abolished this increase. In another experiment in which the same proportionate effect of seminiferous tubules in the absence of LH stimulation was observed, infusion of 1.0 ng/ml LH produced a further increase in the effect of seminiferous tubules upon Leydig cell steroidogenesis. These results suggest that the effect of seminiferous tubules upon Leydig cells is a function not only of the ratio of seminiferous tubules to Leydig cells but is also

modulated by the degree of gonadotrophin stimulation of Leydig cells. In the light of these observations it would appear unlikely that the observed effects are the result of non-specific tissue interactions.

Although seminiferous tubules are known to contain relatively high levels (300-1000 pg/10 cm, Chapters 6-7) of testosterone it has been shown, both by perifusion of seminiferous tubules alone and by calculating the maximum tubular contribution to testosterone in column effluates, that the effects observed cannot be attributed to leakage of testosterone from the tubules. Neither can the effects be explained by the presence of Leydig cells adhering to the tubules, as in these studies minimal amounts of testosterone were produced by seminiferous tubules perifused alone, and no significant binding of 125-I hCG to isolated tubules in vitro was demonstrable.

The ability of seminiferous tubules to stimulate Leydig cell testosterone production was shown to be dependent upon the degree of oLH stimulation of the Leydig cells. At very low levels of LH stimulation the ability of seminiferous tubules to stimulate Leydig cell testosterone production was completely nullified and inhibitory effects were seen, whilst at higher doses of LH the response of the tubules varied in a dose-dependent manner. These results suggest that the seminiferous tubules are sensitive to the degree of gonadotrophin stimulation to which their companion Leydig cells are exposed and that the tubules modify the production of factors which affect the Leydig cells in the light of that gonadotrophin stimulation. The means by which the Leydig cells 'inform' the seminiferous tubules of the presence of the varying degrees of gonadotrophin stimulation they are undergoing, and the subsequent response of the tubules are not

known at this time. However, the presence of a complex interaction between these compartments, involving in all likelihood more than one factor of seminiferous tubular origin, is evidenced by the complex modulation of seminiferous tubular stimulation of Leydig cells. It is apparent that stimulation of Leydig cells by seminiferous tubule factors can be 'switched off' or reversed, as indeed it is in the presence of 0.01ng/ml LH, or increased and decreased in a manner related to but not determined by the fluctuations in peripheral LH stimulation. That is, it would appear that the seminiferous tubules respond to LH by 'reading' a signal from the Leydig cells, and following the receipt of such a signal(?testosterone) are able to modulate the output of factors affecting Leydig cell testosterone production.

It has been shown that altering the flow rate of medium through the perifusion system has no dramatic effects on the response of Leydig cells to co-perifusion with seminiferous tubules. Such studies were initially undertaken as a pilot for the further reduction of flow rate through the system and to investigate the possibilities of scaling down the flow rate, and ultimately the amount of tubules and Leydig cells required for the columns. One of the major drawbacks of the present system is the extensive preparation required to enable each experiment to be performed. For example, each experiment requires 16-24 million Percoll-purified Leydig cells, 800-1200 cms of dissected seminiferous tubules, and 1.5 litres of perifusion medium. Therefore for each experiment up to 24 adult rats are required. The scaling down of the system would allow a greater range of experiments to be attempted since the preparation time would be greatly reduced.

In conclusion, the system described herein provides a novel and powerful tool for the investigation of dynamic interactions between seminiferous tubules and Leydig cells in vitro. These studies have shown that seminiferous tubules, added to Leydig cells in 'physiological' proportions, exert stimulatory effects on Percoll-purified Leydig cells. These effects are modulated by LH stimulation. Future studies using co-perifusion of seminiferous tubules and Leydig cells could be expected to contribute greatly to our understanding of the interactions between these cell types in vivo, since this in vitro system represents the closest approach to the physiological state yet described. Future directions for study involve the co-perifusion of seminiferous tubules with unpurified interstitial cells , investigation of the effects of various hormones(FSH, β-endorphin, oestrogens, oxytocin), investigation of possible inhibitory effects of seminiferous tubules upon Leydig cells and the scaling down of the perifusion system to allow more detailed investigations to be undertaken.

CHAPTER 6

THE INTRATESTICULAR DISTRIBUTION OF TESTOSTERONE DURING DEVELOPMENT AND DURING TESTOSTERONE WITHDRAWAL

6.1: Introduction:

The production of spermatozoa in mammalian species is driven and maintained by testosterone, produced by the interstitial Leydig cells and targeted on the Sertoli cells within the seminiferous tubules. It is known that while spermatogenesis can be qualitatively maintained following hypophysectomy in the presence of low(10-20% normal) intratesticular testosterone levels(Ahmad, Haltmeyer & Eik-Nes, 1973; Cunningham & Huckins, 1979; Buhl, Cornette, Kirton & Kaun, 1982), much higher levels of testosterone are required (in the upper end of the normal range) to maintain quantitatively normal spermatogenesis(Stevens & Steinberger, 1983). In the preceding chapters(3-5) mechanisms for the local control of testosterone production have been shown to exist(See Ch 5), and the literature also supports the existence of locally produced factors which modulate Leydig cell testosterone production(see Chapter 1). Despite extensive evidence for the dependence of spermatogenesis upon testosterone(see Sharpe, 1983), little or nothing is known about the precise role of testosterone in the spermatogenic process.

Studies on isolated seminiferous tubules from the adult rat testis have demonstrated considerable changes in various aspects of Sertoli cell function(see Chapter 1; Parvinen, 1982 or Sharpe 1983 for review). It has been shown that the testosterone content of the seminiferous tubules is greatest at stages VII-VIII(Parvinen & Ruokonen, 1982), which is the acutely androgen-dependent period of the cycle(Sharpe, 1983). Following on from these reports techniques have been developed for the assessment of testosterone distribution throughout the testis, in the seminiferous tubules, interstitial fluid (from which the seminiferous tubules derive their testosterone) and in

whole testis tissue, to provide further insight into the local control of intratesticular testosterone levels, as well as providing basic information on the normal testosterone distribution between these compartments. Therefore the distribution of testosterone was assessed in animals throughout puberty and also following treatments which impaired Leydig cell function(treatment with an antiserum to LH and cryptorchidism). Parts of some of these experiments were performed in collaboration with Dr. R.M. Sharpe.

6.2: Validation of techniques for the measurement of testosterone in seminiferous tubules:

To validate the dissection procedure for isolation of seminiferous tubules the following experiments were performed:

i) hCG binding:

As described previously(Chapter 2) hCG binding to both seminiferous tubules and to Percoll purified Leydig cells was determined. No significant($p>0.1$) hCG binding to seminiferous tubules was demonstrated(99+/-135 c.p.m./10 cm tubules, mean+/-s.d. N = 4) whilst Leydig cells showed significant hCG binding(206,900+/-15,800 c.p.m./10^6cells).

ii) Testosterone diffusion from isolated seminiferous tubules:

Tubules were dissected as described in Chapter 2 by teasing from decapsulated testes at 4°C and incubating 20 x 0.5 cm lengths in 2 mls medium at 21°C for periods of 0, 20, 60 and 240 mins. When compared with the time zero tubule testosterone concentration of 437+/-72 pg/10 cm(mean+/-s.d., n = 4), after incubation at 21°C for 20 mins, values had decreased by over 50%(215+/-71 pg) and by 60 and 240 min had become virtually undetectable(19+/-33 and <5 pg/10 cm respectively); these decreases were matched by corresponding

increases in the testosterone detectable in the incubation medium. However, the experiments described below suggest that by cooling the medium to 4°C diffusion of testosterone from the seminiferous tubules can be largely prevented during the first 60 mins of the dissection procedure.

iii) Comparison of seminiferous tubule testosterone content before and after collagenase treatment:

Tubules dissected from testes either after decapsulation or after decapsulation and collagenase digestion at 32°C for 5 min were extracted and the testosterone content compared. Testosterone levels in isolated seminiferous tubules from undigested testes were not significantly different from those obtained from testes after collagenase digestion in either tubules from control rats (930+/-389 pg/10 cm, n = 8 vs 894+/-198 pg/10 cm, n = 5; teased vs collagenase digested tubules) or those from animals treated with an LH antiserum(262+/-70 pg/10 cm, n = 5 vs 180+/-38 pg/10 cm, n = 6; teased vs collagenase digested tubules).

iv) Comparison of total testis testosterone with tubule testosterone content:

Further evidence to support the supposition that during tubule isolation diffusion of testosterone from the tubules was minimal was provided by calculating the total testicular content of seminiferous tubule and interstitial fluid testosterone and comparing this value with the testosterone content of the contralateral testis from the same animal. Results showed that the testosterone content of seminiferous tubules + interstitial fluid were not significantly different from contralateral testis testosterone values(182+/-84 ng vs 149+/-63 ng, ST + IF vs total

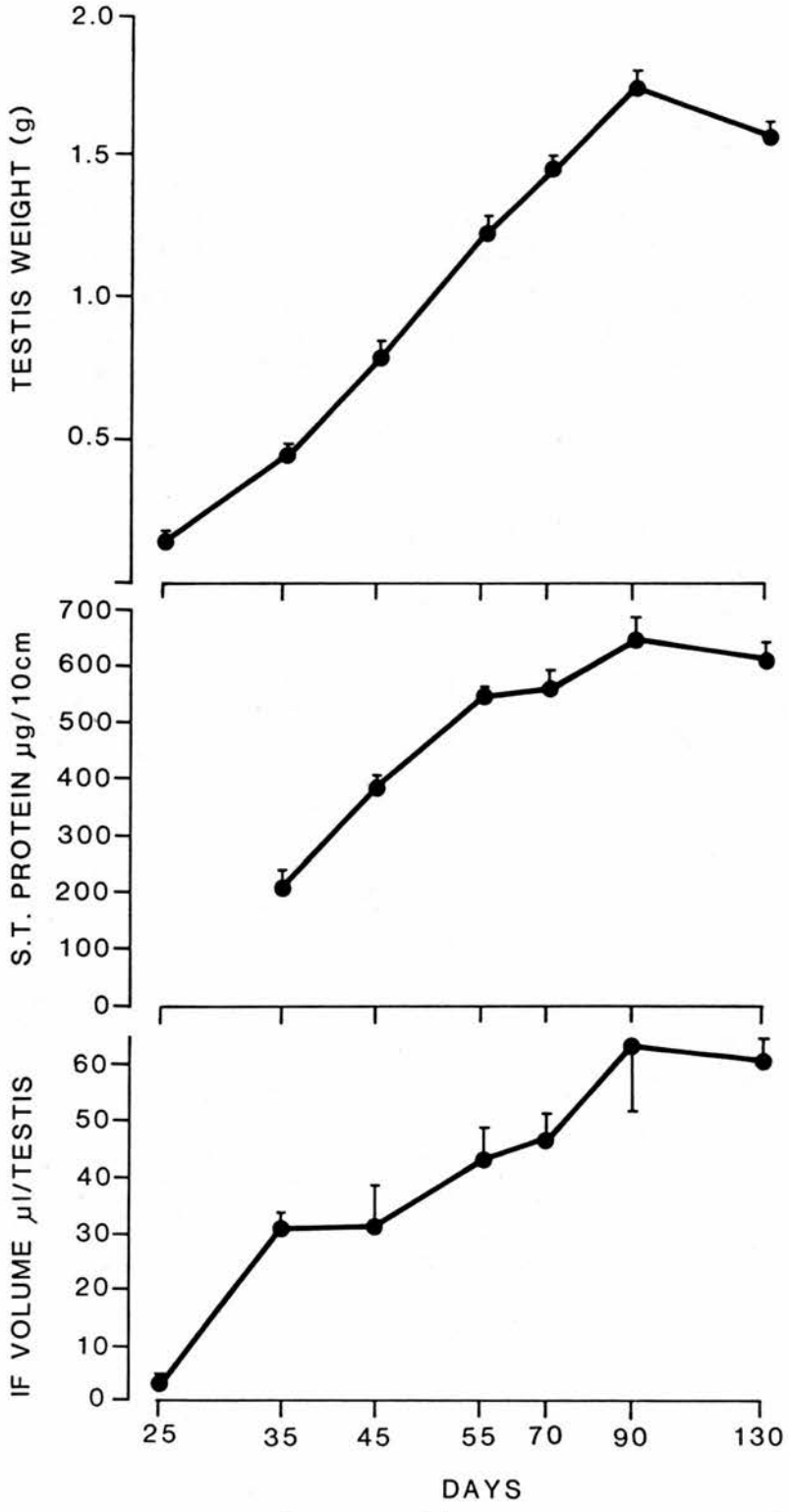


Fig 6.1: Testis weight(Top panel), interstitial fluid(IF) volume (Bottom panel), and seminiferous tubule(S.T.) protein concentration (Centre panel)during maturation in the rat. Values plotted are the mean +/- s.d. of 5 animals per group.

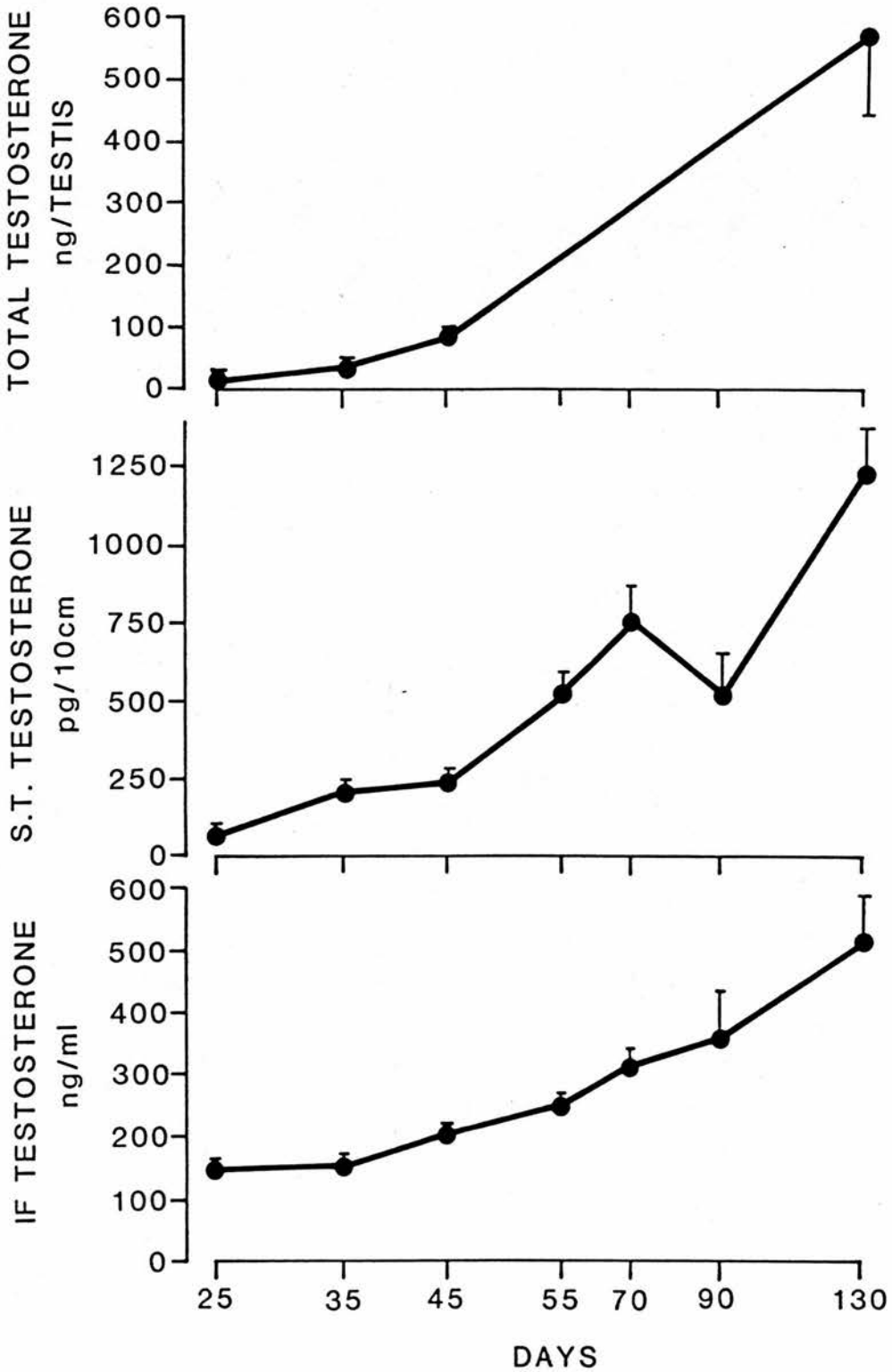


Fig 6.2: Testosterone distribution within the testis during maturation in the rat. Total testis content of testosterone(Top panel), seminiferous tubule(S.T.) testosterone concentration(Middle panel) and interstitial fluid(IF) testosterone concentration(Bottom panel) are shown. Values plotted are the mean +/- s.d. of 5 animals per group.

testis, n = 9).

6.3: The distribution of testis testosterone during normal maturation in the rat:

As a basis to further investigations, and also to test the methods developed and validated above, the following experiment was performed. Groups of rats were killed at 25, 35, 45, 55, 70, 90 & 130 days after birth, and tubule, interstitial fluid, and whole testis content of testosterone measured. Testis weight, recovered interstitial fluid volume and seminiferous tubule protein content were also recorded.

Seminiferous tubule protein content was below the assay detection limit at 25 days of age, values being less than 25 µg/10cm of seminiferous tubules(Fig 6.1). As expected, testicular weight, seminiferous tubule protein content and interstitial fluid volume all increased steadily between 25-90 days, and thereafter tended to plateau(Fig 6.1). Whilst testis weight and seminiferous tubule protein increased in a more or less linear pattern, interstitial fluid volume increased sharply between 25-35 days before assuming a more gradual rate of increase thereafter(Fig 6.1). Total testis testosterone and interstitial fluid testosterone also increased in a linear fashion between 25-130 days(Fig 6.2). However, whilst both seminiferous tubule testosterone and total testis testosterone content were initially low, <50 pg/10cm & <50 ng/testis, respectively, at 25 days of age, interstitial fluid testosterone concentrations were relatively high at this time (approx 140 ng/ml, Fig 6.2). Seminiferous tubule testosterone levels also increased in a linear fashion between days 25-70, although thereafter some fluctuation in values was evident(Fig 6.2). By expressing the seminiferous tubule

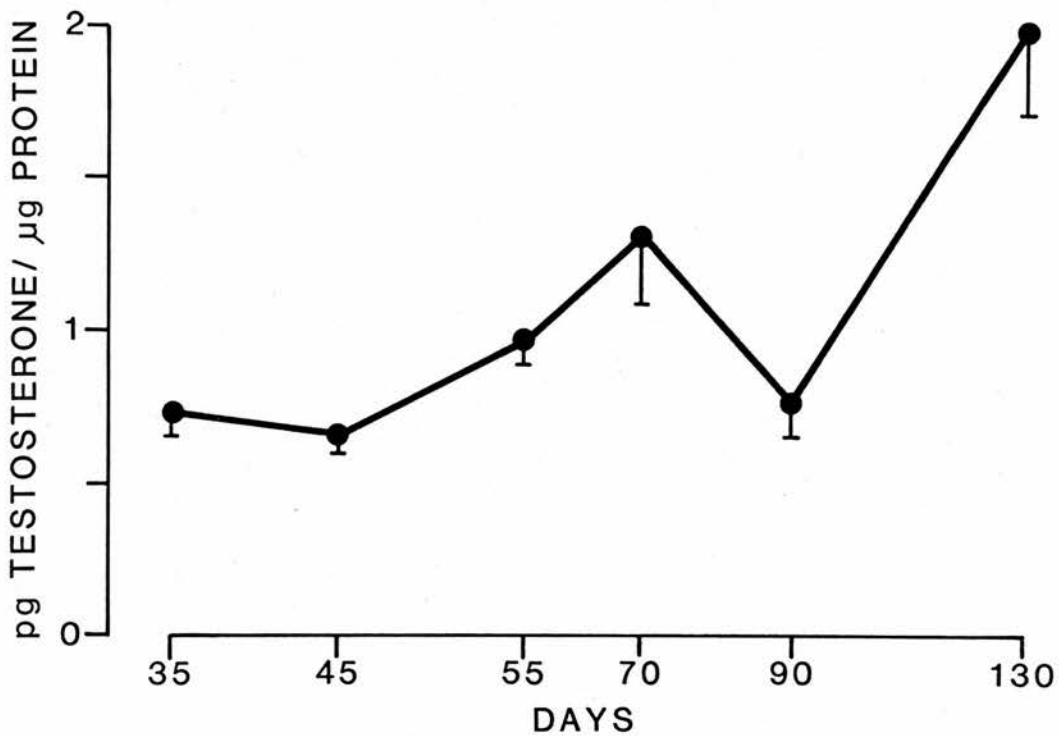


Fig 6.3: Seminiferous tubule testosterone, expressed per µg of seminiferous tubule protein, during maturation in the rat. Values plotted are the mean +/- s.d. of 5 animals per group.

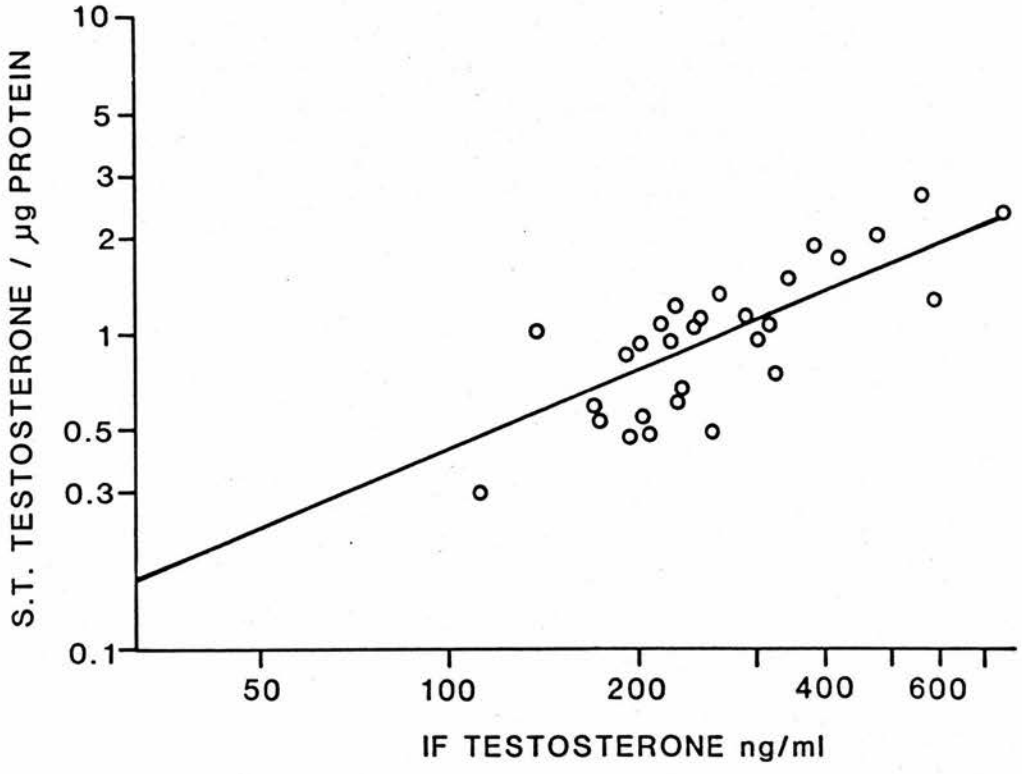


Fig 6.4: Relationship between seminiferous tubule testosterone (expressed/µg protein) and the interstitial fluid testosterone concentration in individual rats of different ages. Values are plotted on a logarithmic scale. The line of best fit is shown(r = 0.825, n = 29).

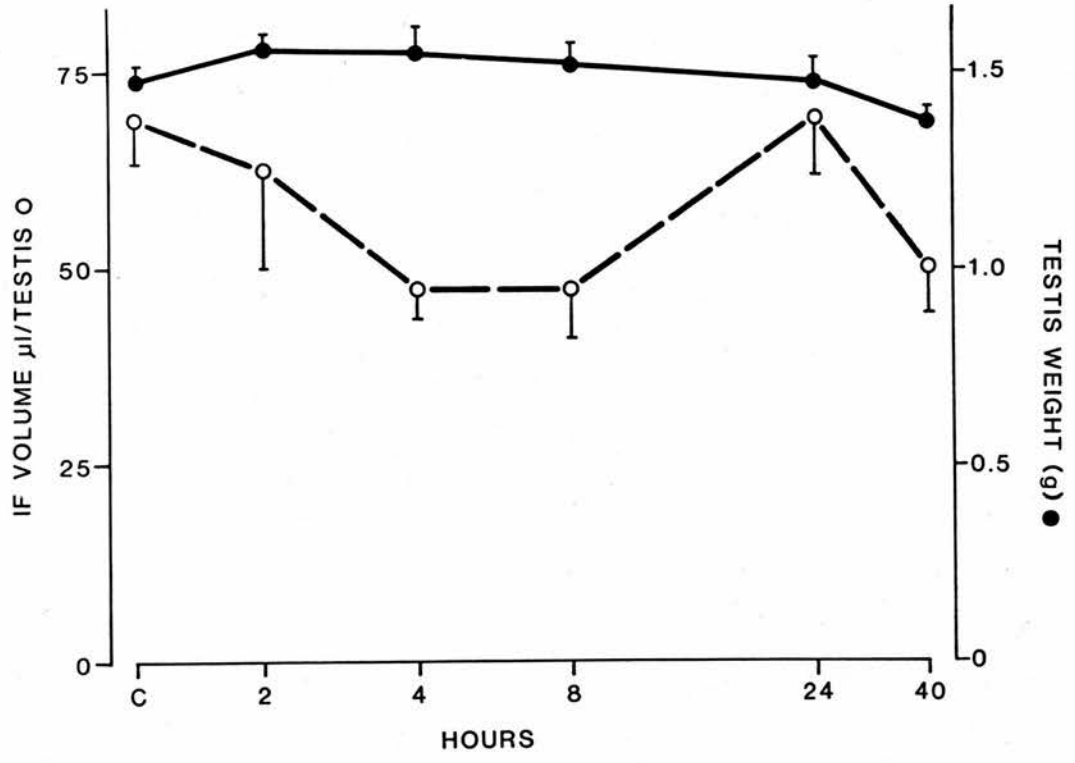


Fig 6.5: Effect of short-term treatment with an antiserum to oLH on the recovered interstitial volume(open symbols) and testicular weight(closed symbols). Values plotted are the mean +/- s.d.(Numbers are as shown for Fig. 6.6), C = control rats.

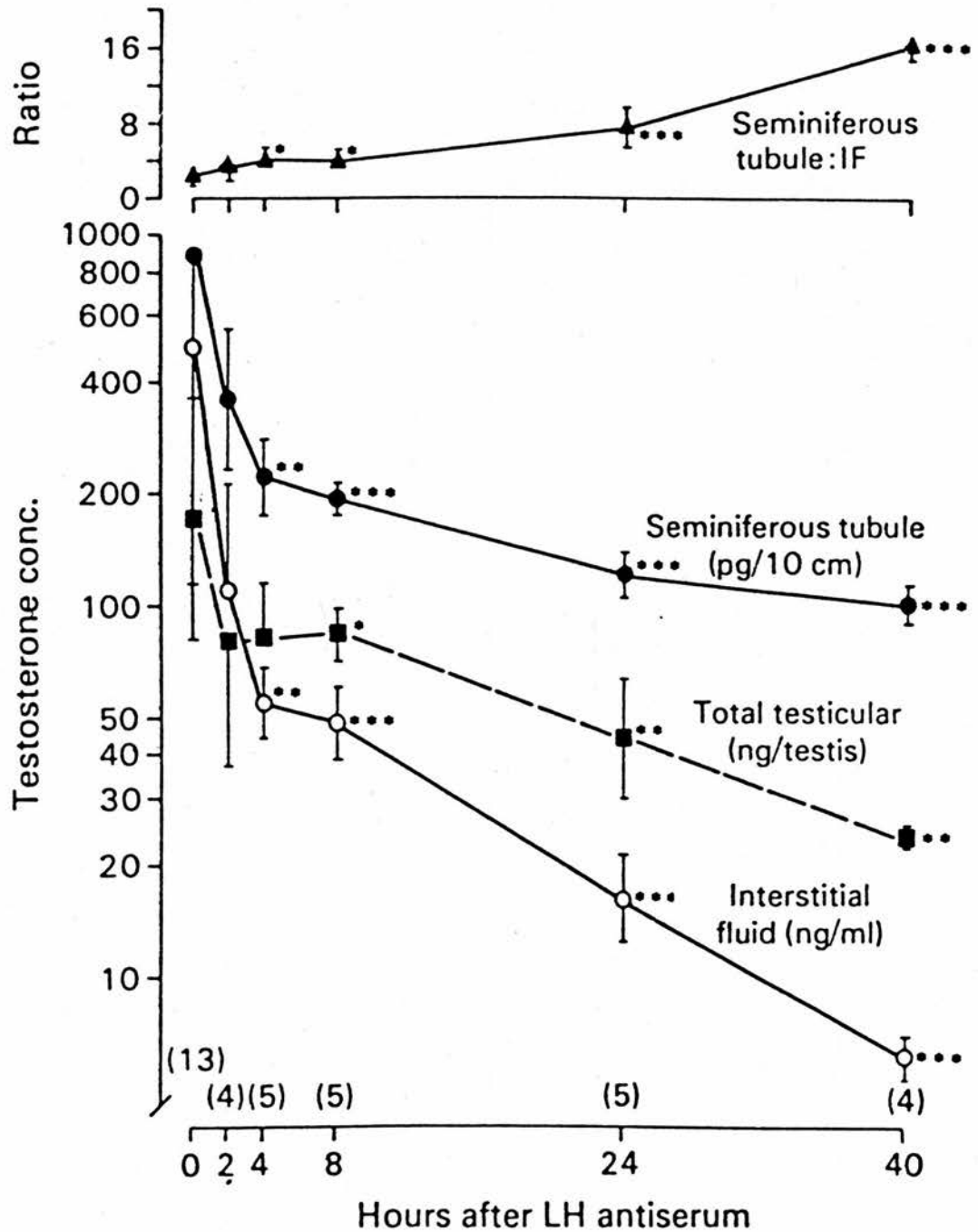


Fig 6.6: The effect of administration of LH antiserum on temporal changes in the concentration of testosterone in testicular interstitial fluid(IF) and seminiferous tubules in relation to the total testicular content of testosterone(lower panel). Testosterone values are plotted on a logarithmic scale and the ratios of testosterone values in the tubules to that in IF are plotted in the top panel. Each point is the mean +/- s.d. for the number of animals shown in parentheses along the bottom axis. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, in comparison with the control group.

testosterone content as a function of the protein content of the tubules it could be seen that throughout development the amount of testosterone present in the tubules/microgram of protein remained relatively constant(Fig 6.3), i.e. between 0.5 & 1.25 pg/μg protein, between days 25-90, although values for older animals(130 days) were somewhat higher(approx 2 pg/μg). To determine if the seminiferous tubule testosterone content and interstitial fluid testosterone concentration in individual animals were related, seminiferous tubule testosterone/μg protein was plotted against the interstitial fluid testosterone concentration(Fig 6.4). The line of best fit for these values was determined by linear regression and the values found to be positively correlated($r = 0.825$, $n = 29$; $p<0.001$).

6.4: Changes in the intratesticular distribution of testosterone following administration of an antiserum to oLH.

6.4.1: Testis weight and interstitial fluid volume:

No significant change($p>0.05$) in either testis weight or recovered interstitial fluid volume was seen during the 40h following administration of a single dose of an LH antiserum to adult rats(Fig 6.5). Interstitial fluid volume decreased at 4-8h post-injection by about 30%(Fig 6.5) but this decrease was not significant, and by 40 h post-injection values had returned to control levels.

6.4.2: Intratesticular testosterone distribution:

Following injection of an antiserum to LH, testosterone concentrations in interstitial fluid fell precipitously and progessively, such that by 4h post treatment, levels were reduced by nearly 90% when compared with controls. By 40 h post treatment interstitial fluid testosterone values were reduced by 99% when compared with control values(Fig 6.6). The seminiferous tubule

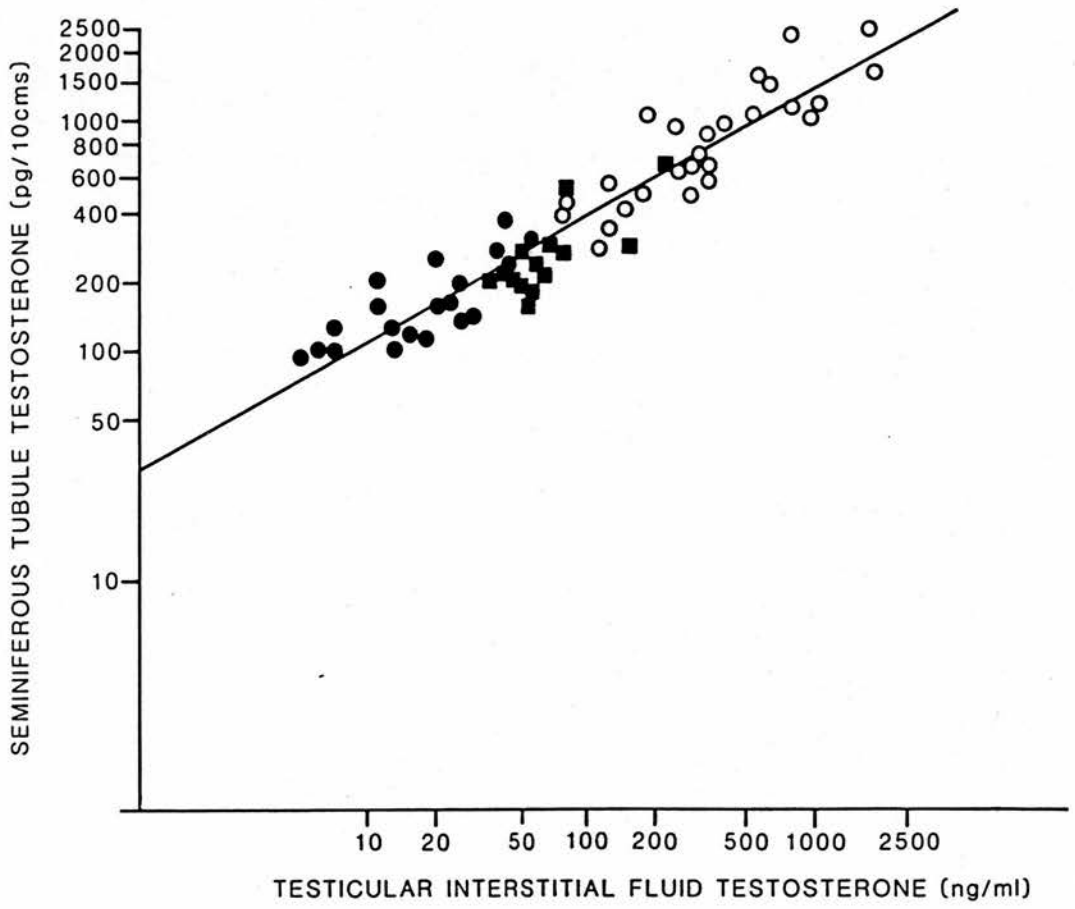


Fig 6.7: Correlation between testosterone concentrations inside the seminiferous tubules with those outside in testicular interstitial fluid in control rats and rats treated with an LH antiserum. Both sets of values are plotted on a logarithmic scale. The solid line is the best fit calculated by regression analysis of the log values ($y = 0.55x + 1.49$, $r = 0.983$). Open symbols are values from control animals, closed symbols are from anti-LH treated rats, of which round symbols are animals treated for between 16-40 h and square symbols for animals treated for between 2-8 h.

concentration of testosterone also fell rapidly to 25% of control values within the first 4 h after treatment, but thereafter fell slowly such that by 40 h after injection with anti-LH, seminiferous tubule testosterone content was still 11% of control values(Fig 6.6). Confirmation that the rate of decline of testosterone concentrations in the two compartments was different was provided by calculating the ratio of the testosterone concentration in seminiferous tubules to that in interstitial fluid. This ratio was significantly($p<0.05$) increased within 4 h of injection, and by 40 h was increased more than 5-fold compared with controls($p<0.001$; Fig 6.6). Measurement of the total testicular testosterone content showed a pattern similar to that of seminiferous tubule testosterone, with more than a 60% reduction by 4 h compared with control values, and values reduced to 11% of controls by 40h. Although by 40 h after anti-LH the reduction in both seminiferous tubule and total testis testosterone was identical, there was a slight discrepancy at 4 h when seminiferous tubule testosterone was reduced by 75%, whilst total testis testosterone was reduced by only 60%.

Using these data, the relationship between the testosterone concentration in seminiferous tubules and interstitial fluid from individual animals was assessed by plotting the values on a log scale(Fig 6.7). Over a wide(200 fold) range of values there was a strong positive correlation between testosterone concentrations in the two compartments($r = 0.983$, $p<0.001$, $n = 60$). The regression line was not, however, indicative of a 1:1 relationship between interstitial fluid testosterone and seminiferous tubule testosterone(Regression equation, $y = 0.55x + 1.49$).

Days post cryptorchidism	Serum hormone concentration ng/ml		
	LH	FSH	TESTOSTERONE
3	56+/-36	488+/-114	5.1+/-5.1
5	68+/-20	362+/-40	3.6+/-1.4
13	55+/-17	490+/-85	4.6+/-2.8

Table 6.1: Serum LH, FSH and testosterone values following experimental induction of unilateral cryptorchidism in the rat. Values are the mean +/- s.d. of 5 animals. No significant($p>0.05$) change in serum concentrations of any of these hormones were observed during the times shown.

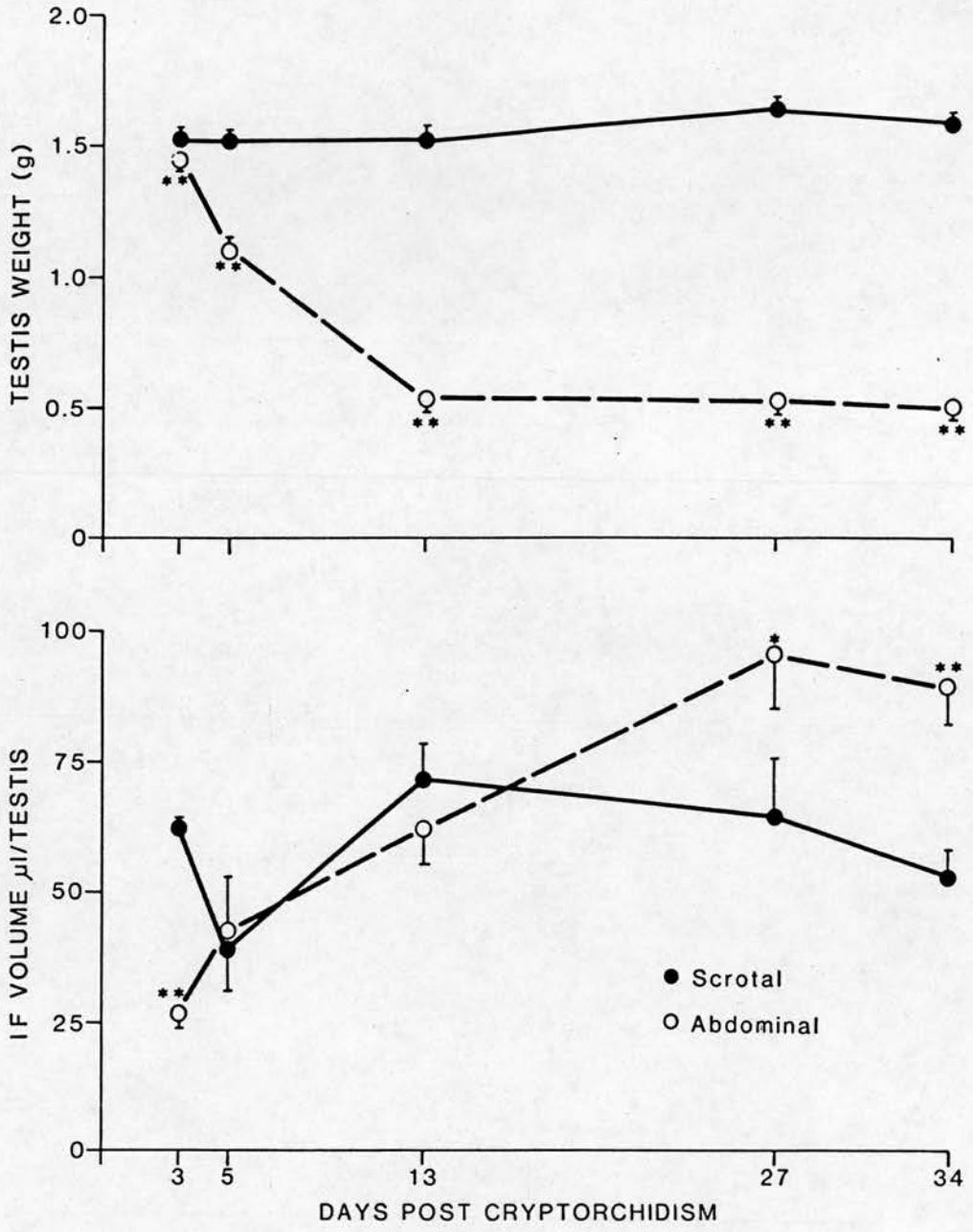


Fig 6.8: Effect of experimental induction of unilateral cryptorchidism on testicular weight(top) and recovered intersitial fluid volume (bottom) from scrotal testes(closed symbols) and abdominal testes(open symbols). Mean +/- s.d. Numbers are shown along the bottom axis of the lower panel of Fig. 6.9. * $p<0.05$, ** $p<0.001$ significant difference between scrotal and abdominal testes calculated by paired t-test.

6.5: Intratesticular testosterone distribution following experimental induction of cryptorchidism.

Further studies were undertaken, using unilaterally cryptorchid rats, to determine the effects of this treatment on the intratesticular distribution of testosterone.

6.5.1: Serum hormone concentrations:

Serum concentrations of LH, FSH and testosterone remained unchanged for up to 13 days after experimental induction of unilateral cryptochidism(Table 6.1).

6.5.2: Testis weight, interstitial fluid volume and seminiferous tubule protein content:

No change in the seminiferous tubule protein concentration was seen until 13 days after induction of cryptorchidism, when tubule protein content fell to approximately 50% of values from the contralateral testis(Data not shown). Testis weight, however, was significantly reduced within 3 days of induction of experimental cryptorchidism($p<0.001$, Fig 6.8), although it was still 93% of control values. By 5 days post operation, abdominal testis weight had fallen to 73% of the weight of the contralateral scrotal testis, and by 13 days and thereafter the abdominal testis weights were reduced to 34% of contralateral testis values(Fig 6.8). The interstitial fluid volume fell significantly by 3 days post-operation, recovered between 5-13 days and was elevated at 27 & 34 days post operation(Fig 6.8).

6.5.3: Intratesticular distribution of testosterone following experimental induction of cryptorchidism:

Seminiferous tubule, interstitial fluid and whole testis testosterone levels in the abdominal testis were reduced by

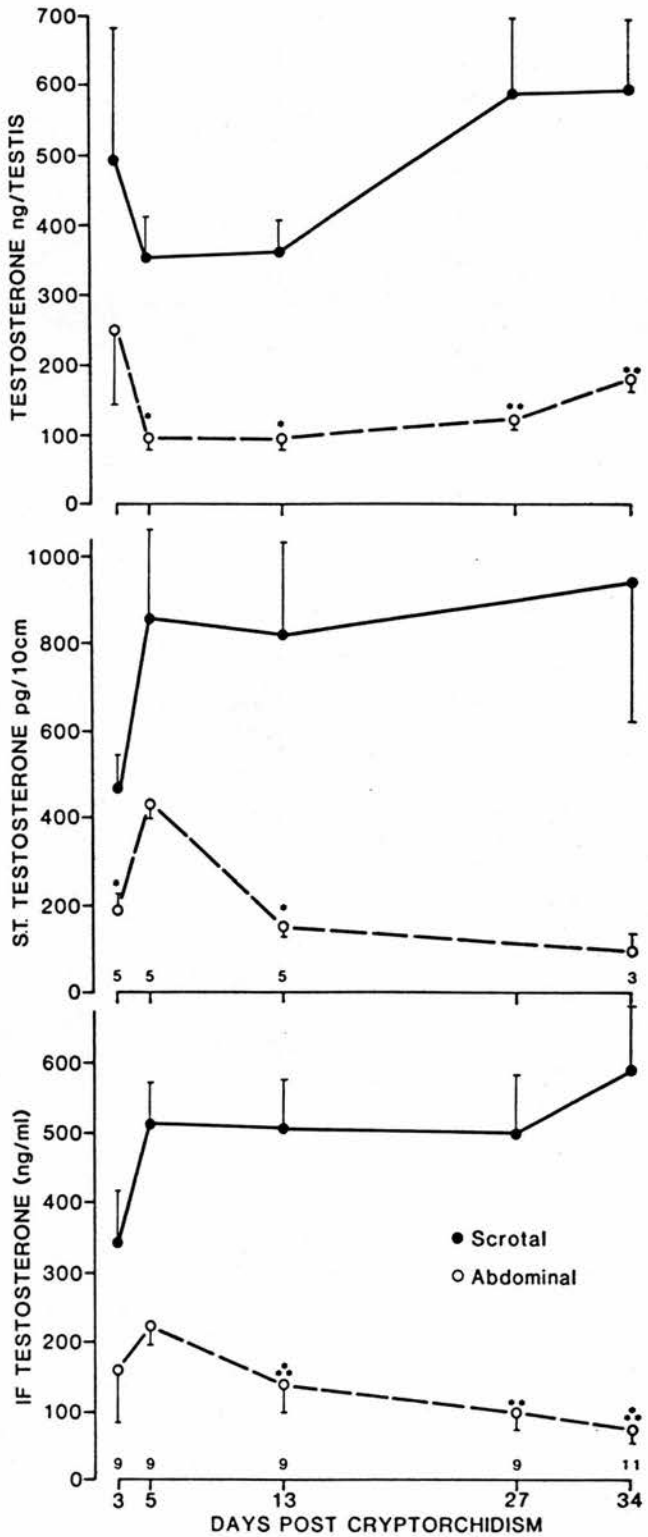


Fig 6.9: Effect of experimental induction of unilateral cryptorchidism on the intratesticular distribution of testosterone. Total testis testosterone(top), seminiferous tubule(S.T.) testosterone(middle) and interstitial fluid(IF) testosterone concentrations(bottom) are shown. Mean +/- s.d. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ significant differences between scrotal(closed circles) and abdominal(open circles) testes calculated by paired t-test. Numbers of animals are shown above the bottom axis of the middle panel for both total testis and S.T. testosterone and along the lower panel for IF testosterone.

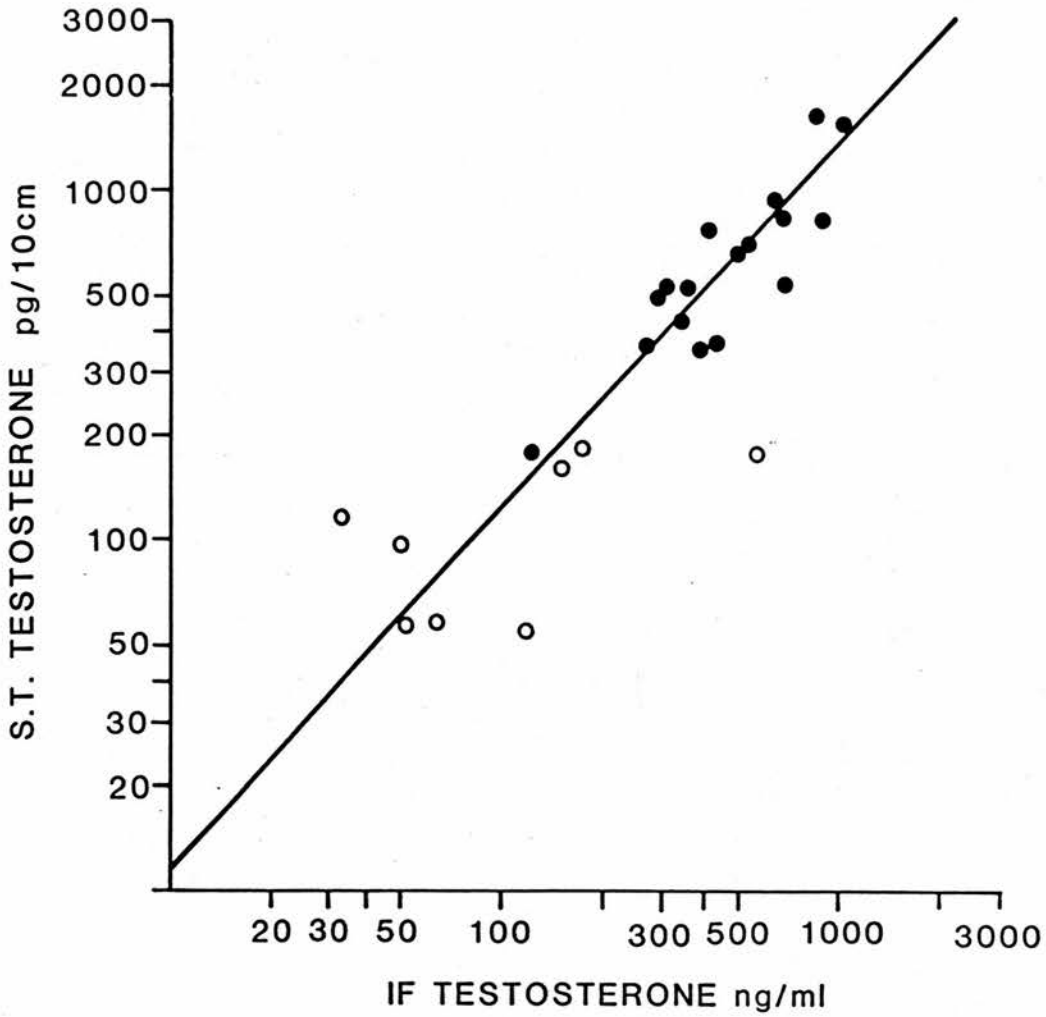


Fig 6.10: Correlation between testosterone concentrations inside the seminiferous tubules with those outside in testicular interstitial fluid in scrotal(closed symbols) and abdominal(open symbols) testes from unilaterally cryptorchid rats. Both values are plotted on logarithmic scales. The solid line is the best fit calculated by regression analysis of the log values from the scrotal testes ($y = 1.5x + 44$, $r = 0.841$).

approximately 50% within 3 days of induction of cryptorchidism when compared with the contralateral scrotal testis(Fig 6.9). However, due to considerable variation between animals, only the difference in seminiferous tubule testosterone was significant at this time($p<0.05$). By 5 days post-operation, seminiferous tubule testosterone levels were not further reduced, when compared with the contralateral scrotal testes, although thereafter values decreased to 20-25% of values for the scrotal testis at 13 and 34 days post-operation,($p<0.05$). Interstitial fluid testosterone values followed a similar pattern as did values for the total testis testosterone(Fig 6.9). These values were again used to determine the relationship, under these conditions, between interstitial fluid and seminiferous tubule testosterone levels. Using a linear regression program, the line of best fit was described($y = 1.5x - 44$) for the values from scrotal testes, and the correlation between seminiferous tubule and interstitial fluid testosterone levels calculated(0.841). Values from the abdominal testes did not fit this relationship, but were found to fit a different linear regression($r = 0.71$, $y = 0.34x + 80$) suggesting that a linear relationship between these parameters was maintained following cryptorchidism.

6.6: Discussion:

These techniques allow the distribution of a major intratesticular hormone, testosterone, within the testis to be assessed for the first time. However before any valid conclusions can be drawn from the data presented above, the validity of the techniques used must be firmly established.

In a study of the inter-compartmental distribution of testosterone within the testis, the greatest concern is that

inaccuracies in the measurement of this hormone may occur either due to diffusion of the hormone from the tissue or between compartments between the time of death and the time of isolation of the respective tissues or fluids. The validity of the technique used for the isolation of interstitial fluid over 16 h at 4°C has been reported elsewhere(Chapter 2), as have the techniques for extraction of the tissues(de Jong, Hey & Van der Molen, 1974) and for the assaying of samples(Sharpe & Bartlett, 1985). The major concern which must be addressed here is the possibility that testosterone diffuses out of the seminiferous tubules during the dissection and isolation procedures used. It is generally assumed that in vivo, testosteone is distributed throughout the testicular tissues by passive diffusion, and the techniques used for the isolation of seminiferous tubules were therefore carefully validated.

Experiments with testes which had been dispersed with collagenase at 32°C showed that diffusion from the tubules under these conditions was minimal(see 6.2 above). However, once tubules were dissected into 0.5 cm lengths, diffusion at 21°C was rapid, with 50% of testosterone being lost within 20 mins. Cooling to 4°C markedly retarded this process, and it is assumed that within the 8-15 minutes required to dissect the tubules from an individual testis, diffusion is neglible. This agrees with earlier findings by Parvinen and Ruokonen(1982), and is further supported by the observation that by summing the testicular interstitial fluid testosterone content(calculated from the volume of interstitial fluid in the testis and the testosterone concentration in that fluid) and the seminiferous tubule testosterone content/testis (calculated by multiplying tubule testosterone content by total testicular tubule length, based on 1240 cm/gm testis; Wing &

Christensen, 1982) the values thus obtained were not significantly different from the measured testosterone content of the contralateral testis(see 6.2 above). This calculation was only carried out for anti-LH treated animals, since both age and cryptochidism may alter the total testicular length of seminiferous tubules, as may other treatments inducing tubular damage(see Chapter 7).

As a further test of the validity of these results, hCG binding to seminiferous tubules teased from undigested testes was assessed. No significant binding to isolated seminiferous tubules was demonstrated(see 6.2 above).

The results obtained for tubule testosterone contents in this study are higher that those previously reported(Podesta & Rivarola, 1974), probably as a result of the precautions taken here to minimise diffusion. Other studies(Ruokonen, Vihko & Niemi, 1973; de Jong et al, 1974; Comhaire & Vermeulen, 1976) are difficult to compare since testosterone concentrations were expressed per unit weight or per ml of seminiferous tubular fluid. Values obtained above for seminiferous tubule testosterone, which ranged in controls from 80-2500pg/10cm are also generally higher than those obtained by Parvinen & Ruokonen(1982), whose methods were largely followed. However, by performing stage dissections, these authors prolong the dissection time(2-3 hrs) and also dissect the seminiferous tubules into shorter lengths(2 mm), both of which would increase the possibility of diffusion of testosterone from the tubules.

As a further assessment of the validity of the techniques and also to provide basic information, the intratesticular distribution of testosterone was determined throughout puberty, and during early adult life. Whilst the results(Figs 6.1-3) are much as would be expected,

with all of the parameters measured increasing throughout development and reaching a plateau thereafter, it should be noted that, although seminiferous tubule and testicular testosterone levels were low during early development, interstitial fluid testosterone levels were already approaching adult values(150 ng/ml) by 25 days post-partum. Furthermore, throughout development, the seminiferous tubular testosterone/protein ratio remained more or less constant(around 0.5-1.2 pg testosterone/μg protein, Fig 6.3). The seminiferous tubule testosterone concentration was shown to be positively correlated with the concentration in interstitial fluid(Fig 6.4), which would seem to confirm that seminiferous tubule testosterone levels are determined by the extratubular testosterone concentration(See below).

Treatment of adult rats with an antiserum to LH(See 6.4 above) resulted in low levels of testosterone within all testicular compartments(Fig 6.6). However, following chemical destruction of the Leydig cells(see Chapter 7), intratesticular levels of testosterone are reduced to below the limit of detection of the assay system used(<2ng/ml in interstitial fluid, <2ng/testis, <25-50pg/10 cm seminiferous tubules). Therefore, in the animals treated with anti-LH the possibility that testicular testosterone concentrations are partially sustained by local factors remains. Recent studies(Sharpe & Cooper, 1984) have identified a factor in rat testicular interstitial fluid which markedly stimulates Leydig cell testosterone secretion, and, in the animals treated with anti-LH described above, levels of this interstitial fluid factor were raised following treatment(Sharpe & Bartlett, 1985). The levels of this factor are also elevated following EDS treatment and the induction of cryptorchidism(Sharpe, Kerr, Fraser & Bartlett, 1985, submitted).

That local factors may support Leydig cell testosterone production is further supported by the evidence showing that even at 40 h following anti-LH treatment, there were significant amounts of testosterone present within the testis. Such observations also agree with data obtained from hypophysectomized animals which show that at three days following removal of the pituitary, testosterone levels within testicular interstitial fluid are low but measurable(46 ng/ml) suggesting that, in the absence of LH stimulation, Leydig cells continue to produce testosterone, possibly under the influence of local regulatory factors(Sharpe, Doogan & Cooper, 1982).

Another factor potentially affecting the distribution of testosterone within the testis is that the seminiferous tubules may preferentially retain testosterone. This is supported by the observation that, following treatment with an antiserum to LH, the ratio between seminiferous tubule and interstitial fluid testosterone levels increased as the interstitial fluid testosterone levels fell(Fig 6.6). Theoretically, these findings could be explained by the presence of a high-affinity androgen-binding protein(ABP) within the seminiferous tubules(e.g. Hansson et al, 1976b), but the reported levels of this factor(Ritzen, Boitani, Parvinen, French & Feldman, 1982) would only account for a minute proportion of the testosterone present in the tubules. Preliminary evidence suggests that a low-affinity, high capacity androgen-binding factor may be present in the seminiferous tubules(Parvinen, 1984) which may explain these observations. That the relationship between interstitial fluid and seminiferous tubule testosterone levels was not determined solely by the interstitial fluid testosterone concentration was shown by the changing relationship between these two parameters in different

treatment groups(Figs 6.4, 6.7, 6.10). Thus the seminiferous tubule testosterone level was not related to the interstitial fluid testosterone concentration on a one to one basis and this was particularly evident when values were towards the low end of the normal range or below the normal range(Fig 6.7). This suggests that when intratesticular testosterone levels fall, mechanisms are activated which conserve the tubular testosterone content, presumably to maintain testosterone levels sufficiently to permit complete spermatogenesis to continue.

This study has also shown marked differences in the testosterone content of testes located within the scrotum and abdomen of cryptorchid animals(Fig 6.9), differences which were associated with marked changes in testicular weight(Fig 6.8). As the serum LH and FSH levels remained unchanged up to 13 days following unilateral induction of cryptochidism(See Table 6.1), the question arises as to how the reduced levels of testosterone output by the Leydig cells in the abdominal testis can be explained.

It has been shown that, following experimental induction of cryptorchidism, marked changes in Leydig cell morphology occur (Kerr, Rich & de Kretser, 1979a,b; Rich, Kerr & de Kretser, 1979) including increased cellular volume and hypertrophy of the smooth endoplasmic reticulum and Golgi apparatus, two of the organelles involved in steroidogenesis. The *in vivo* response of bilaterally cryptorchid animals to exogenous LH or hCG is reduced when compared with controls whilst *in vitro* the testes are hyper-responsive to LH(Kerr *et al*, 1979a; Rich & de Kretser, 1979; see also Chapter 1). Similarly, isolated Leydig cells from the abdominal testes of unilaterally cryptorchid rats are hyper-responsive to LH when

compared *in vitro* with control cells(Sharpe, Cooper & Doogan, 1984). Therefore the reduction in testosterone output seen in these conditions does not seem to be explained by impairment of Leydig cell testosterone output in response to LH stimulation. However, following injection of hCG into bilaterally crytorchid rats, it has been shown that the uptake of hCG *in vivo* into the cryptorchid testis is reduced by up to 70%, when compared with controls, probably as a result of decreased blood flow to the cryptorchid testis(Sharpe, 1983). Therefore, it would appear that the reduction in testosterone levels within the abdominal testis seen in unilaterally cryptorchid rats, is a result of reduced levels of LH reaching the Leydig cells within that testis. This observation also suggests that whilst local factors may play a significant role in the regulation and maintenance of testicular testosterone concentrations, the presence of near normal LH stimulation is essential if such mechanisms are to be completely effective, since in the absence of such stimulation the testis cannot compensate solely by increasing the levels of 'local regulators'. In the light of this observation, it should be noted that the factor(s) present in testicular interstitial fluid which stimulates Leydig cell testosterone secretion is far more effective in the presence of hCG than in its absence(Sharpe & Cooper, 1984), suggesting that it is a synergistic agent which complements LH action rather than replacing it.

In conclusion, methods for analysis of the intratesticular distribution of testosterone have been established, validated and applied to normal developing rats and those in which testosterone levels are lowered. The results suggest that during periods of low testosterone supply, seminiferous tubule testosterone levels are

maintained either by the sequestering of testosterone within the seminiferous tubules or by the action of local factors which stimulate Leydig cell testosterone production, or by a combination of these factors. These methods will provide a useful tool for the future assessment of the role of testosterone in the control of spermatogenesis(see Chapter 7), and of the relative roles of LH and local factors in the maintenance of local concentrations of testosterone.

CHAPTER 7

THE USE OF SPECIFIC TOXINS IN THE ELUCIDATION OF TESTICULAR FUNCTION

7.1: Introduction:

Further to the studies described above(Chapter 6), investigations into the use of specific cellular toxins were carried out. A number of chemicals thought to have specific testicular cell targets have now been identified, such as: methoxyacetic acid(MAA) which is thought to target specifically upon the primary spermatocytes(Foster, Creasy, Foster, Thomas, Cook & Gangolli, 1983; Foster, Creasy, Foster & Gray, 1984), some phthalic acid esters, which are thought to specifically impair Sertoli cell function (Foster, Thomas, Cook & Gangolli, 1980; Foster, Foster, Cook, Thomas & Gangolli, 1982; Creasy, Foster & Foster, 1983), and an alkane sulphonic ester, ethane dimethane sulphonate(EDS) which is thought to impair Leydig cell function(Morris, 1985; Bu'Lock & Jones, 1976; Bu'Lock & Jackson, 1971).

Methoxyacetic acid(MAA) has been shown to be the active agent reponsible for the toxic effects of the ethylene glycol monoethyl and monomethyl esters in the rat(Foster et al, 1984), and these toxins appear to initially affect primary spermatocytes undergoing post-zygotene meiotic maturation and division(Foster et al, 1984). This compound could therefore provide a useful model for the investigation of the effects of disruption of Sertoli cell interactions with a specific germ cell population.

A number of phthalate esters have been shown to have deleterious effects on testicular morphology(Creasy, Foster & Foster, 1983). Of these Di-n-pentyl phthalate has been established as the most effective agent, with respect to the toxic actions described(Creasy et al, 1983). The phthalates have been shown to increase urinary excretion of zinc and to reduce the testicular content of this metal(Foster,

Thomas, Cook & Gangolli, 1980). The morphological effects of these compounds have also been described. Thus, within 3 h of a single oral dose of di-n-pentyl phthalate the early and late spermatocytes are displaced towards the lumen of the seminiferous tubules and there is extensive vacuolation of the Sertoli cell cytoplasm and, by 6 h after treatment, both this displacement of early and late spermatocytes and the degree of Sertoli cell vacuolation was more marked(Creasy et al, 1983). As treatment progressed there was a gradual depletion of germinal cells from all tubules leaving a Sertoli cell matrix with a few necrotic spermatocytes and occasional normal spermatogonia(Creasy et al, 1983). In the light of these studies, and of other studies suggesting that the active metabolites are the de-esterified phthalates, a pilot study using mono-2-ethylhexyl phthalate(MEHP) to disrupt tubular function was undertaken, and the effects of this compound upon the intratesticular distribution of testosterone, testicular weight and serum testosterone and gonadotrophins were determined.

The antifertility effects in rodent species of a number of alkane sulphonic esters are well documented(Jackson, Fox & Craig, 1961, Cooper & Jackson, 1970). Of the substances investigated, one in particular, ethane dimethane sulphonate(EDS), has been shown to specifically impair the endocrine function of the Leydig cells (Jackson, 1973). Other studies(Jackson & Jackson, 1984) have suggested that treatment of fertile rats with EDS results in spermatogenic disruption and temporary loss of fertility due to Leydig cell dysfunction.

In adult rats, EDS has been shown to suppress testicular

synthesis of testosterone and its precursors in vitro(Bu'Lock & Jackson, 1971), and it also markedly decreases the synthesis of cyclic AMP in response to hCG stimulation(Bu'Lock & Jones, 1976). Studies in vivo have shown a marked reduction of serum testosterone levels within 24 h of EDS treatment(Morris & McCluckie, 1979), and parallel increases in plasma gonadotrophin levels(Jackson & Morris, 1977; Morris & Jackson, 1978), suggesting an alteration of the feedback signals from the testis to the pituitary. All of these effects occur within 24 hours of a single EDS treatment. However, levels of circulating reproductive hormones return to within the normal range over a similar time period to the return of fertility, i.e. 6-9 weeks after treatment with EDS(Cooper & Jackson, 1961; Bu'Lock & Jackson, 1975; Jackson & Morris, 1977; Morris & McCluckie, 1979). As suggested by these authors, impairment of parameters of Leydig cell function implies that EDS acts, either directly or indirectly, by impairment of Leydig cell function.

Despite these numerous studies providing evidence for an action of EDS on spermatogenesis via a direct effect on the Leydig cells there is no convincing information on the effects of EDS on the morphological and functional status of the intratesticular environment.

In these studies, two testicular toxins, methoxyacetic acid and MEHP were used in an attempt to disrupt testicular function over a short time period(2 days). Also, further to studies by Kerr, Donachie and Rommerts(1985b) which have shown that EDS specifically destroys Leydig cells in the adult rat, an in depth study of the effects of such destruction of Leydig cells on the intratesticular distribution of testosterone and the maintenance of seminiferous

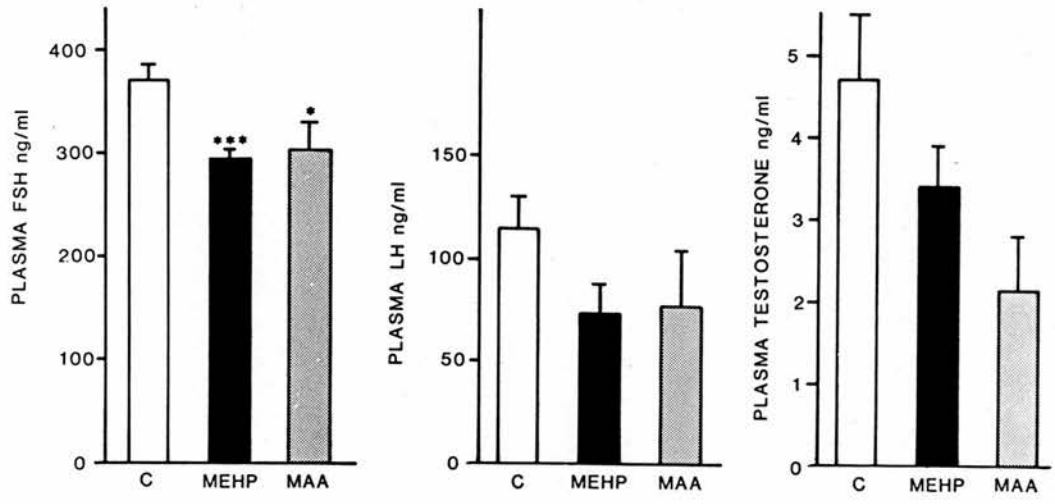


Fig 7.1: Effect of a single dose of MEHP(n = 9) or MAA(n = 3) on FSH(left), LH(centre) and testosterone(right) levels in plasma recovered from adult male rats 2 days after treatment. * $p<0.05$, *** $p<0.001$ when compared with control values(n = 8), Means +/- s.d. (Student's t-test).

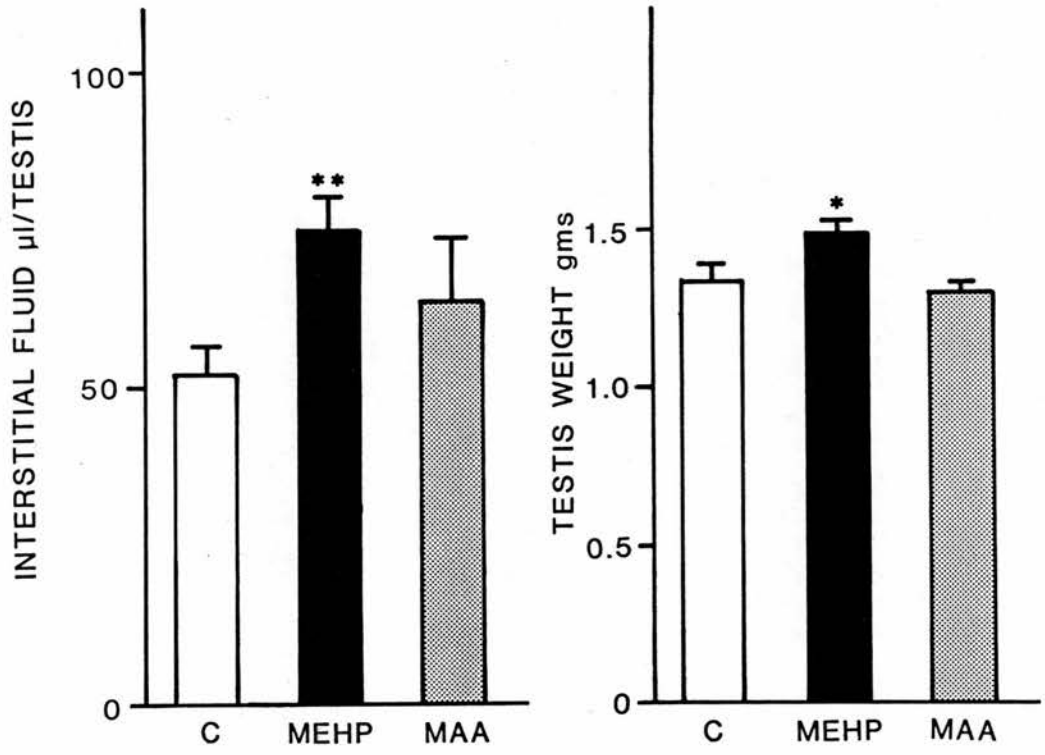


Fig 7.2: Effect of a single dose of MEHP(n = 9) or MAA(n = 3) on the recovered interstitial fluid volume(left) and testis weight(right) of animals two days after treatment. * $p<0.05$, ** $p<0.01$ when compared with control values(n = 8), Means +/- s.d.(Student's t-test).

tubule and interstitial tissue morphology and function was performed. These studies were performed in collaboration with Dr. J.B. Kerr and Dr. R.M. Sharpe.

7.2: Effects of Methoxy acetic acid(MAA) and mono-2-ethylhexyl phthalate on testicular function:

The effect of a single dose of MAA and MEHP given by oral gavage on the testicular function of adult male rats was assessed. Two days after treatment 8 controls, 9 MEHP-treated and 3 MAA-treated rats were killed and plasma LH, FSH and testosterone levels measured as well as the levels of testosterone in interstitial fluid, seminiferous tubules and whole testes. Testicular weight and interstitial fluid volume were also recorded.

a) Plasma LH, FSH and testosterone:

Two days following a single dose of either MAA or MEHP, plasma testosterone and LH levels were decreased, although, in neither case was the effect significant(Fig 7.1). Plasma FSH was significantly decreased in both MAA-treated animals($p<0.05$) and in those treated with MEHP($p<0.01$) within two days(Fig 7.1).

b) Testis weight and interstitial fluid volume:

Treatment with MAA produced no changes in either testis weight or the recovered interstitial fluid volume(Fig 7.2). Treatment with MEHP, however, produced a slight but significant increase in both testicular wieght(11%, $p<0.05$) and the recovered interstitial fluid volume(44%, $p<0.01$) when compared with controls.

c) Testicular distribution of testosterone:

Treatment with MAA caused a 26% reduction in seminiferous tubule testosterone content, a 34% reduction in interstitial fluid testosterone concentrations and also a 9% reduction in the total

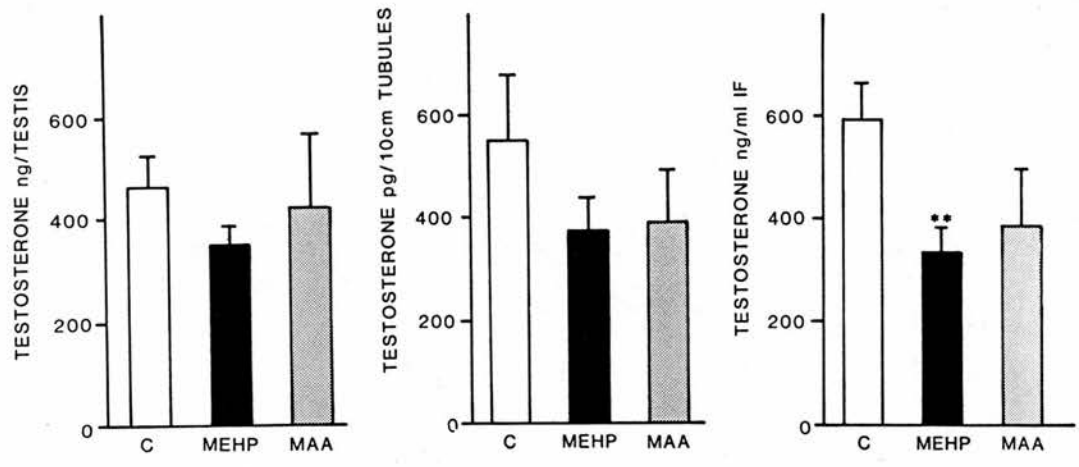


Fig 7.3: Effect of a single dose of MEHP(n = 9) or MAA(n = 3) on total testicular content of testosterone(left), seminiferous tubule(TUBULES) testosterone content(centre) or interstitial fluid(IF) testosterone concentration(right) two days after treatment. ** $p<0.01$ when compared with control values(n = 8), Means +/- s.d.(Student's t-test).

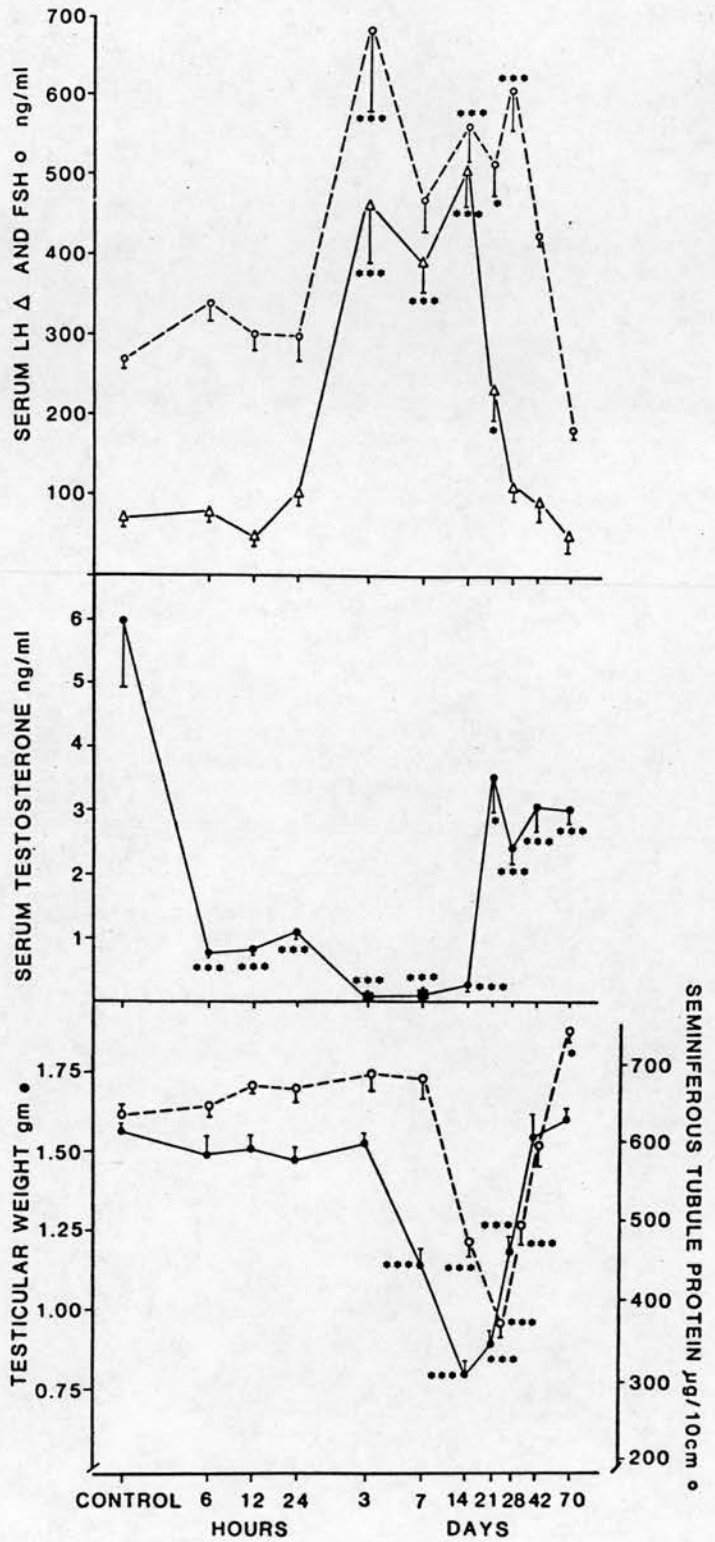


Fig 7.4: Temporal changes in(top) the serum levels of LH and FSH and (middle)the serum levels of testosterone in relation to(bottom) testicular weight and seminiferous tubule protein content following a single injection of EDS into adult rats. * $p<0.025$, ** $p<0.01$, *** $p<0.001$, in comparison with control values. Mean+/-s.e.(n as described for Fig 7.5).

testicular content of testosterone; however none of these effects were significantly different from control values. Treatment with MEHP produced similar reductions in both seminiferous tubule testosterone content(reduced by 30%) and interstitial fluid testosterone concentration(reduced by 42%) but produced a greater decrease in total testicular testosterone content when compared with controls(reduced by 22%). However, only the decrease in interstitial fluid testosterone was statistically significant($p<0.01$) when compared with controls(Fig 7.3).

7.3: Effects of EDS on testicular morphology and function:

a) Serum hormones:

Serum testosterone fell rapidly and significantly($p<0.005$) within 6h of treatment to less than 15% of control values(Fig 7.4). At 12 and 24h no further decrease was observed, but at three days after EDS administration there was a further reduction in serum testosterone levels which fell to below the limit of detection of the assay (<0.05ng/ml), and remained undetectable until two weeks post EDS, when a slight recovery was seen(mean values approx 0.3ng/ml). By 3 weeks and thereafter up to 10 weeks, serum testosterone levels returned to within the normal range(1.0-15ng/ml) although the mean levels were still significantly($p<0.005$) reduced compared with controls.

There were no significant($p>0.05$) changes in serum LH and FSH up to 24h following EDS treatment, but between 24 h and 3 days the concentrations of both hormones increased significantly($p<0.005$), reaching 650 and 250% of their respective control values(Fig 7.4). Thereafter, serum LH levels remained elevated to a similar degree (570-650% above controls)up to 2 weeks after EDS treatment, fell to 330% of control values at 3 weeks, and returned to control levels by 4

weeks and remained so at 6 and 10 weeks post EDS. In contrast, serum FSH levels were increased significantly($p<0.005$) for a longer period, up to 4 weeks, following EDS treatment, and did not return to control levels until between 6 and 10 weeks(Fig 7.4).

b) Testis weight and seminiferous tubule protein:

While testis weight is accepted to provide a general index of testicular damage, seminiferous tubule protein content was also measured to provide a further index of tubule integrity.

Whilst the protein content of isolated seminiferous tubules changed in a similar manner to the testicular weight after EDS treatment there were differences in the time scale of these changes. Thus, at 7 days after EDS treatment, testicular weight was significantly decreased($p<0.005$) whilst there was no significant change in seminiferous tubule protein content(Fig 7.4).

By 2 weeks post-EDS, testis weight had reached its lowest values, being only 50% of control values, whilst seminiferous tubule protein content showed its first significant decrease at this time and did not reach its lowest value until three weeks post EDS, when it represented 57% of control values, and at which time testicular weight had begun to recover. Between 3 and 10 weeks there was a significant recovery of both testicular weight and seminiferous tubule protein content, such that by 6 weeks testicular weight had fully recovered whilst at 10 weeks after EDS treatment seminiferous tubule protein was significantly($p<0.025$) elevated above control values.

c) Interstitial fluid volume:

Within 6 h of EDS treatment the recovered interstitial fluid volume fell signifcantly($p<0.005$) to 30% of control values, and by 12 h had reached less than 12.5% of control values(9 vs 73

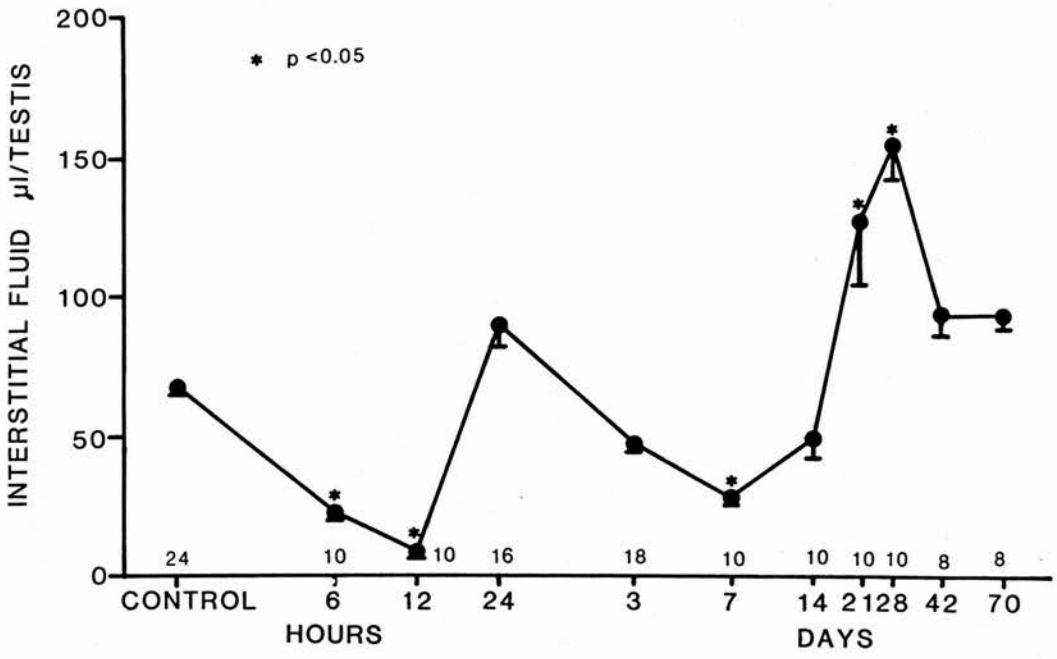


Fig 7.5: Temporal changes in the recovered volume of interstitial fluid from the testes of animals following a single injection of EDS. * $p<0.005$ in comparison with control values. Numbers placed above the 'x' axis represent the numbers of animals per group. Mean+/-s.e.

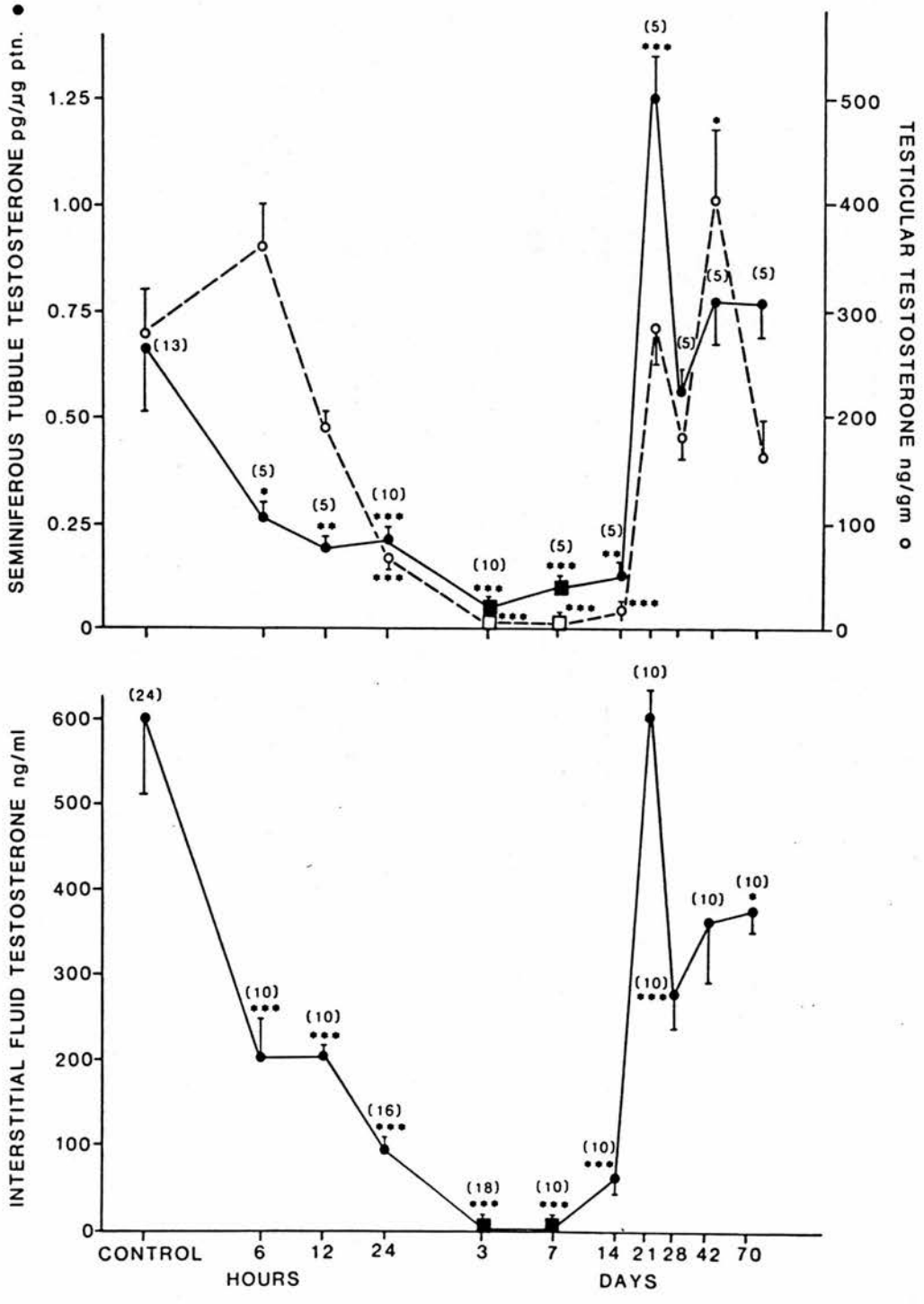


Fig 7.6: Temporal changes in the concentration of testosterone in(top) isolated seminiferous tubules and the whole testis and in(bottom) testicular intersitial fluid following a single injection of EDS into adult rats. In both panels, square symbols indicate values below the limit of detection(see text). * $p<0.025$, ** $p<0.01$, *** $p<0.005$, in comparison with control values(Mean+/-s.e., Numbers are shown in brackets).

µl/testis, Fig 7.5). Thereafter, however, the recovered interstitial fluid volume returned to near normal values, i.e. 45% above controls at 24 h($p<0.005$ vs 12 h values), and 71% of controls at 3 days($p<0.005$ vs 24 h values) after EDS treatment, although neither of these values were significantly different from control values. By 7 days post EDS treatment the recovered interstitial fluid volume was again significantly lower than control values($p<0.005$, Fig 7.5). Between 3-14 days post EDS treatment there was no significant change in interstitial fluid volume compared with controls, although between 2-3 weeks after EDS treatment recovered interstitial fluid volume more than doubled($p<0.005$ vs controls & 2 week values). By 4 weeks after EDS treatment the interstitial fluid volume was 212% of control values($p<0.005$, Fig 7.5). Thereafter, by 6 weeks after treatment with EDS the recovered interstitial fluid volume had fallen significantly($p<0.005$ vs 4 weeks) to reach control values and remained at this level until 10 weeks post EDS treatment(approx 90 µl/testis, Fig 7.5).

d) Intratesticular distribution of testosterone:

The total testicular content of testosterone per gram of tissue was unaltered at 6h after EDS treatment despite a significant($p<0.05$-0.005)fall in serum, interstitial fluid and seminiferous tubule levels of testosterone(Fig 7.6). Between 6 and 12h post EDS there was a significant($p<0.025$) fall in testicular testosterone, and between 12h and 3 days after EDS the values fell progressively, to reach levels at or below the limit of detection by 3 days(<2.0ng/gm). Between 3 and 7 days post EDS treatment, testicular testosterone was barely detectable, but by 2 weeks there was the return of small but measurable amounts of testosterone in the testis, and thereafter the

content rose rapidly to reach about 250ng/gm. Between 3-4 weeks there was a transitory decrease in testosterone content per gram of tissue but at six weeks post EDS, the testosterone content of the testis was significantly($p<0.025$) increased compared with control values, before it fell again slightly by 10 weeks ($p<0.005$ vs 6 week values).

Interstitial fluid levels of testosterone fell significantly by 6h post EDS, but, unlike serum testosterone levels which fell by >85% within 6h, interstitial fluid testosterone fell by only 67%. Between 6 and 12h after treatment there was no further fall in the interstitial fluid testosterone concentration, but between 12 and 24h, levels fell by 50%(Fig 7.6). Within 3 days after injection of EDS, interstitial fluid levels of testosterone were undetectable(<2ng/ml) and remained so until 2 weeks when concentrations of around 50 ng/ml were measured. By 3 weeks post EDS treatment interstitial fluid levels of testosterone were increased to values comparable to those in controls, but thereafter fell again to about 50% of the control levels and then recovered slightly during weeks 6 and 10.

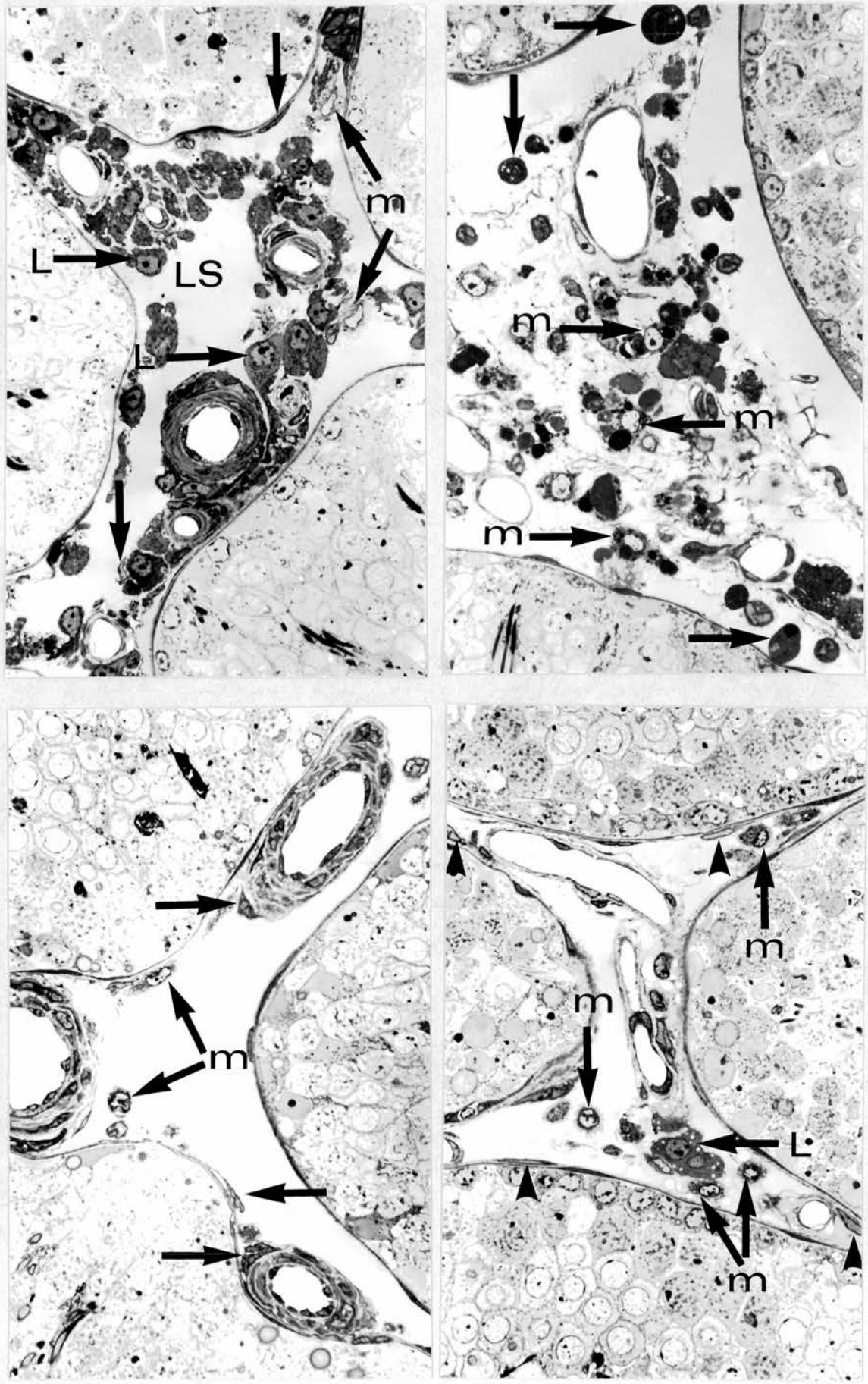
Because of the observed change in seminiferous tubule protein content, seminiferous tubule testosterone measurements have been expressed as pg of testosterone per µg of tubular protein(Fig 7.6). Seminiferous tubule testosterone levels fell rapidly over the first 6h following EDS treatment, but then remained stable between 6 and 24h post EDS. Beyond 24h seminiferous tubule testosterone levels fell to become undetectable by 3 days post EDS treatment(<21-50pg/10cm, <0.05-0.1pg/µg protein) but by 2 weeks levels were again detectable (0.125pg/µg protein). Three weeks after EDS treatment seminiferous tubule testosterone concentrations had increased to nearly twice the control values(1.2pg/µg protein) but by 4 weeks were once again within

Fig 7.7: Top left: Vehicle treated rat, showing typical morphology of the interstitial tissue, which contains Leydig cells(L), macrophages(M), endothelial nuclei(arrows) and the interstitial lymphatic space(LS)(Mag x 930).

Top right: One day after EDS treatment, illustrating fragmented interstitial cells, pyknotic bodies(arrows), macrophages(M) containing large cytoplasmic inclusions and interstitial fibrillar material(Mag x 930).

Bottom left: Seven days after EDS treatment, when Leydig cells have disappeared, leaving macrophages(M) and endothelial or fibroblast nuclei(arrows)(Mag x 930).

Bottom right: Two weeks after EDS treatment, illustrating Leydig cells with cytoplasmic lipid droplets(L), macrophages(M) and spindle-shaped interstitial cells(arrows)(Mag x 930).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the control range and remained so at 6-10 weeks.

e) Testicular histology:

i) Leydig cells and interstitium:

Leydig cells in vehicle-treated animals showed normal adult histology, with polygonal or irregular nuclei containing a small nucleolus. The cytoplasm was granular in appearance reflecting the abundant numbers of mitochondria(Fig 7.7). Macrophages were also present within the interstitial tissue, characterized by their pale-staining nuclei and cytoplasm containing small granules, representing their lysosomal components.

By 24h after EDS treatment many of the Leydig cells showed signs of disintegration and fragmentation, and numerous dense pyknotic bodies were present both within the interstitial space and within the cytoplasm of interstitial macrophages(Fig 7.7). Leydig cells which had not yet broken down completely showed evidence of morphological alterations involving vesiculation of the cytoplasm. Other Leydig cells or their fragmented parts appeared to be enclosed by macrophage cytoplasm, suggesting that they were being phagocytozed by the macrophages. The interstitial space also contained random depositions of fibrillar material, a feature not seen in control animals.

At 3 and 7 days following EDS injection the interstitial space contained no histologically identifiable Leydig cells or pyknotic structures, nor were there any cells present which were recognisable as either damaged or atrophied Leydig cells(Fig 7.7). Interstitial macrophages were still evident, although there were no longer any basophilic inclusions within their cytoplasm.

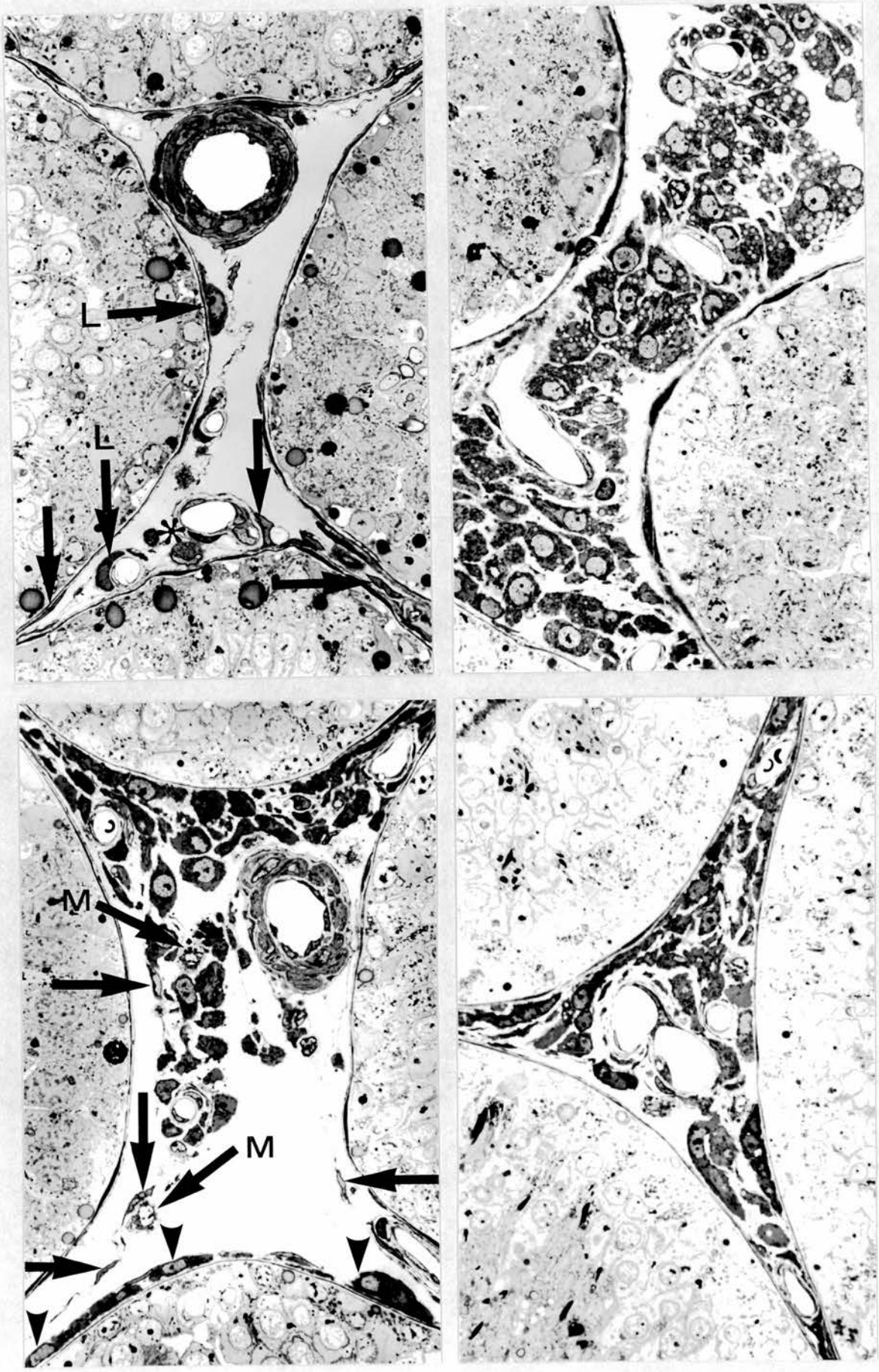
By 2 and 3 weeks after EDS administration the interstitium contained very small numbers of Leydig cells in perivascular or

Fig 7.8: Top left: Three weeks after EDS treatment, showing perivascular and peritubular Leydig cells(L), mononucleated spherical cells(asterisk) and numerous elongated interstitial cells(arrows) (Mag x 930).

Top right: Four weeks after EDS treatment, illustrating abundant foetal-like Leydig cells with many pale-staining cytoplasmic lipid inclusions(Mag x 930).

Bottom left: Six weeks after EDS treatment, showing numerous Leydig cells. Macrophages are shown(M), together with peritubular Leydig cells(arrowheads) and fusiform interstitial cells(arrows) (Mag x 930).

Bottom right: Ten weeks after EDS treatment, illustrating normal adult-type morphology of the interstitial tissue(Mag x 930).


L
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peritubular locations(Figs 7.7-7.8). In contrast to the typically irregular shape of Leydig cells in the control testes, the Leydig cells at 2 and 3 weeks post-EDS exhibited smoothly contoured shapes with few cytoplasmic granules. The interstitium also contained numerous elongated or spindle-shaped cells although their identity could not be established by light microscopy(Fig 7.8).

A remarkable feature of the interstitial tissue 4 weeks post-EDS was the abdundance of a new population of Leydig cells(Fig 7.8). In some sections the Leydig cells often filled the interstitial space in large clusters, yet in contrast, other areas of interstitial tissue contained no Leydig cells. By light microscopy the Leydig cells were very similar to foetal rat Leydig cells(see Chapter 1), exhibiting spherical nuclei with little or no heterochromatin and numerous cytoplasmic lipid inclusions.

At 6 weeks after EDS it was occasionally possible to identify 'foetal-type' Leydig cells although the majority showed morphological features intermediate between foetal-like and the classical adult-type Leydig cell. Thus, the nucleus was often spherical and lacked significant deposits of heterochromatin(similar to foetal Leyidg cells), whilst the cyptoplasm more closely resembled the adult structure, lipid inclusions being rarely observed(Fig 7.8). Peritubular interstitial cells associated with the seminiferous tubules also included elongated Leydig cells, possibly enclosed within the thin cytoplasmic extensions of endothelial cells. Macrophages, mononucleated cells and fusiform-shaped interstitial cells resembling endothelial tissue were commonly observed within the interstitial area.

At 10 weeks after EDS treatment the interstitial space and its

Fig 7.9: Top left: Vehicle treated rat, illustrating normal seminiferous epithelium at stage VIII. Note Sertoli cell nuclei(S), primary spermatocytes(P) and round(R) and elongated spermatids (Mag x 930).

Top right: Three days after EDS treatment, showing two basally situated degenerating bodies(arrows)(Mag x 930).

Bottom left: Seven days after EDS treatment, illustrating increased frequency of degenerating cells(arrows) within stage VII and VIII seminiferous tubules(Mag x 930).

Bottom right: Seven days after EDS treatment, showing a stage X seminiferous tubule with retention of sperm heads and flagella (arrows), and a degenerating spermatid(S)(Mag x 930).

R
S
P
S

VIII
VII

S

population of interstitial cells were similar in appearance to the normal adult rat(Fig 7.8). The Leydig cells were predominantly adult in appearance, with features characteristic of the adult population of Leydig cells as described above.

ii) Seminiferous epithelium:

Up to two days after EDS treatment, light microscope examination of the seminiferous epithelium showed no detectable morphological changes from controls(Fig 7.9). The first alteration in the normal composition of the seminiferous epithelium was seen at 3 days after treatment, when a few seminiferous tubules at stages VII and VIII of the spermatogenic cycle were found to contain degenerating primary spermatocytes(Fig 7.9), and occasional focal aggregations of vacuoles within the basal area of the Sertoli cell cytoplasm. However, these structural alterations were not evident in the majority of tubules at these stages.

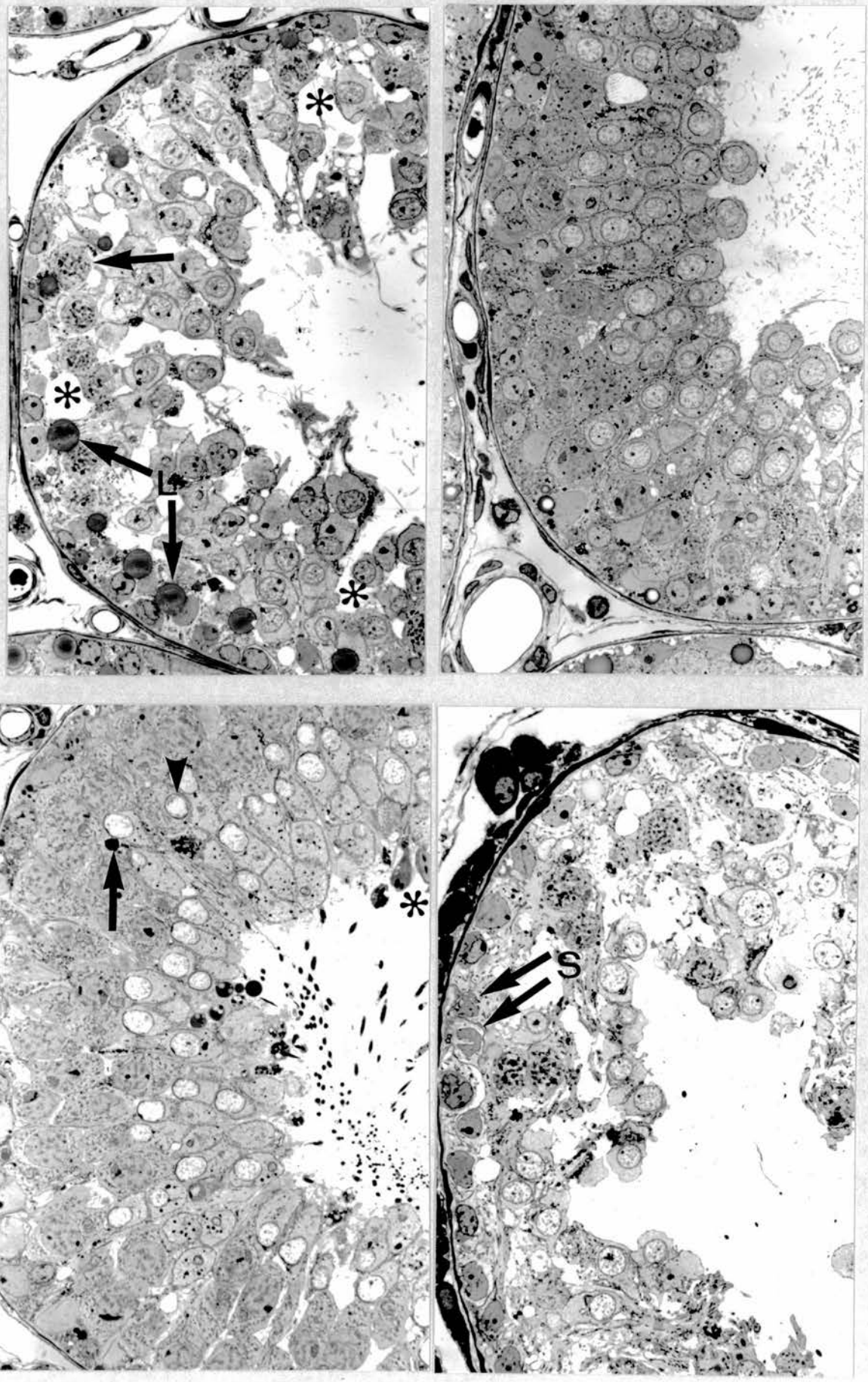
At 7 days after EDS, some stage VII seminiferous tubules showed degenerating primary spermatocytes(Fig 7.9), together with basally situated vacuoles in the region of the Sertoli cell tight junctions. Occasionally the whole Sertoli cell cytoplasmic body was altered, containing irregular vacuoles which were empty or 'watery' in appearance. The majority of stage VII tubules showed qualitatively normal spermatogenesis with no indication of cellular damage. In stages VIII-IX basal Sertoli cell vacuoles and degenerating germ cells were apparent, together with a number of retained spermatid heads and tails often deeply embedded within the body of the Sertoli cell. There was also evidence of abnormal patterns of spermiation during stage VIII of the cycle(Fig 7.9). Within stages X-XI occasional undigested remnants of residual bodies were observed within the basal Sertoli

Fig 7.10: Top left: Two weeks after EDS treatment, illustrating impairment of spermatogenesis at stage II. Note large lipid inclusions(L), extracellular spaces(asterisks) and few primary spermatocytes(arrow)(Mag x 930).

Top right: Three weeks after EDS treatment, showing absence of mature spermatids in a stage VII tubule(Mag x 930).

Bottom left: Four weeks after EDS treatment, showing substantial recovery of spermatogenesis at stage VIII-IX. Note pyknotic body(arrow), abnormal spermatid nucleus(arrowhead) and aberrant residual body(asterisk)(Mag x 930).

Bottom right: Four weeks after EDS treatment, illustrating disruption of spermatogenesis. Note irregular Sertoli cell nuclei(S)(Mag x 930).


S

cell cytoplasm. Despite the range of the changes seen at this time, the degenerative changes were not extensive as the majority of tubules were qualitatively normal in appearance.

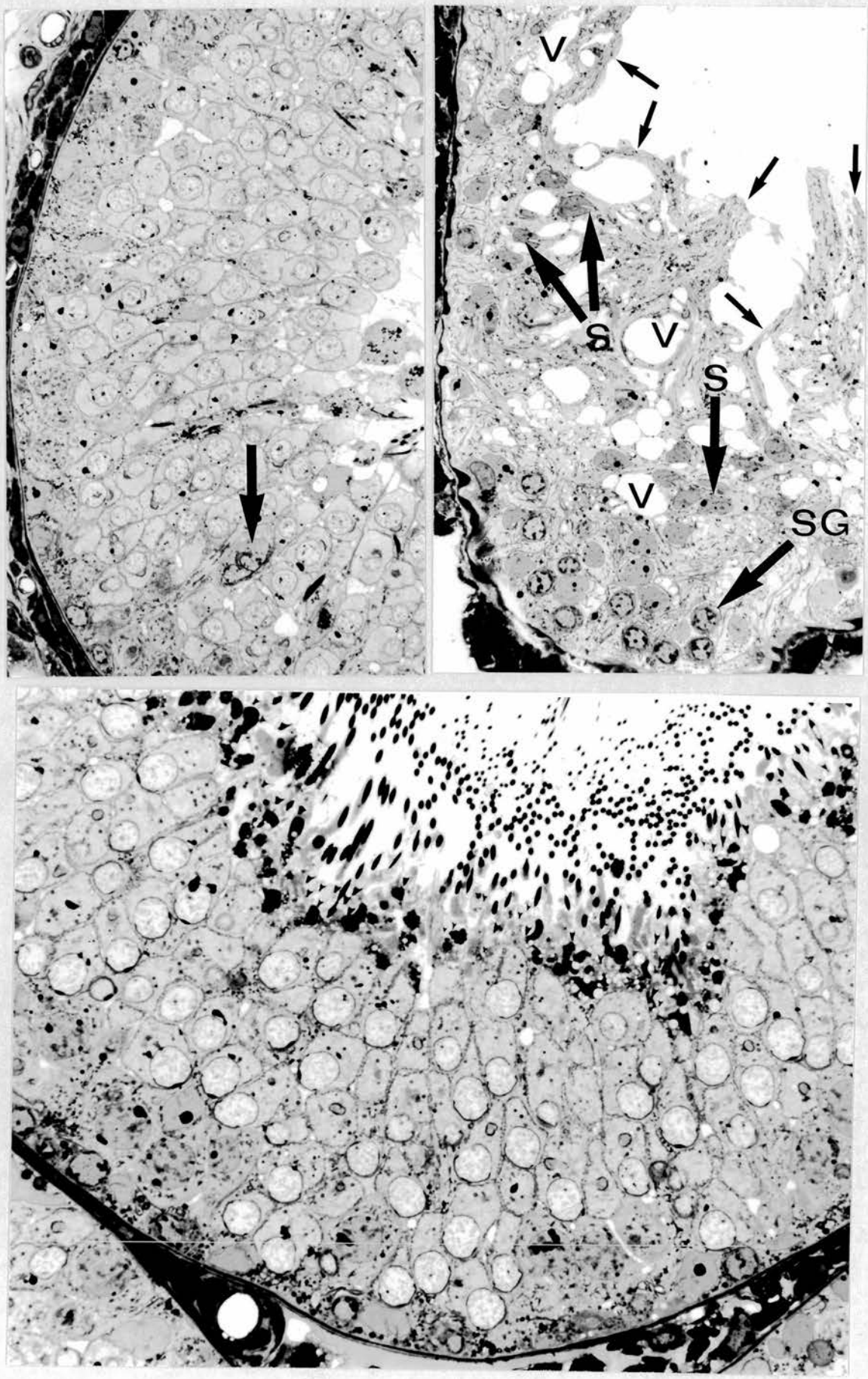
Two weeks post-EDS, histological alterations to the seminiferous epithelium were both more common and involved a wider range of cell types. Disruption of spermatogenesis occurred in stages VII-II of the spermatogenic cycle, although stages III-VI exhibited no qualitatively detectable changes by light microscopy. Tubules at stages VII-VIII showed similar cellular alterations to those described at 7 days. Mature elongated spermatids were either absent or, if present, showed aberrant morphology and orientation in the adluminal regions of the epithelium. During stages IX-XIV a variety of histological changes were seen which included the formation of basally-located vacuoles, wide extracellular spaces, increased lipid inclusions, degenerating primary spermatocytes and loss or degeneration of elongating spermatids. The most severely disrupted tubules, at stages I-II, showed a very substantial or total depletion of elongating spermatids (Fig 7.10), and an apparent reduction in primary spermatocytes together with epithelial vacuoles, extracellular spaces and accumulation of large lipid inclusions.

By the third week following EDS treatment, depletion of the mature generation of elongating spermatids, with increased numbers of basal Sertoli cell lipid droplets and occasional degenerating primary spermatocytes could be seen at stages III-VIII of the spermatogenic cycle. At times, seminiferous tubules at stage VI-VII of the spermatogenic cycle appeared normal except for the absence of the mature generation of spermatids(Fig 7.10). Abnormal retention of sperm heads and residual cytoplasm were often seen at stages IX-XI, together

Fig 7.11: Top left: Six weeks after EDS treatment, illustrating substantial restoration of spermatogenesis at stage III. A degenerating germ cell is shown(arrow)(Mag x 930).

Top right: Ten weeks after EDS treatment, showing a severely disrupted seminiferous tubule. Note pleomorphic Sertoli cell nuclei(S), vacuoles(V), extensions of Sertoli cell cytoplasm(arrows) and spermatogonia(SG)(Mag x 930).

Bottom: Ten weeks after EDS treatment, showing qualitatively normal spermatogenesis at stage VIII(Mag x 1300).


V
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SG

with basal degenerating germ cells and vacuoles. Stages XII-II appeared qualitatively normal by light microscopy.

By 4 weeks after EDS injection most stages of the spermatogenic cycle showed some degree of damage similar to the pattern of disruption seen during weeks 2-3. However restoration of qualitatively normal seminiferous epithelial architecture was occasionally observed at stages VII-VIII(Fig 7.10) in which the epithelium showed considerable depth due to the appearance of several germ cell generations. The few structural abnormalities noted were dense pyknotic bodies, occasional extraction of spermatid nuclear chromatin and unusual configurations of spermatid residual cytoplasm. Otherwise many tubules at these stages appeared normal. In a very small number of tubules, severe germ cell damage was noted(Fig 7.10), with substantial loss of germ cells. These collapsed seminiferous tubules also contained abnormal pleomorphic profiles of Sertoli cell nuclei and disorganization of the Sertoli cell cytoplasm.

Six weeks after EDS injection, spermatogenesis began to show signs of recovery, the vacuoles and lipids associated previously with the Sertoli cell cytoplasm having largely disappeared by this time. Stages I-II appeared normal except for a reduction in the normally plentiful numbers of elongated spermatids, whilst stages III-IV(Fig 7.11) and VII-IX contained reduced numbers of elongating spermatids, and stage IX also lacked normal numbers of residual bodies. Stages X-XI contained fragments of residual bodies not normally seen at these stages. Stages XII-XIV showed occasional abnormalities of flagella and cytoplasmic tails of elongating spermatids. Stages IV-V were still severely damaged, totally lacking in spermatids, with only occasional primary spermatocytes and spermatogonia present.

By 10 weeks the majority of seminiferous tubules were fully expanded, with a normal seminiferous epithelium(Fig 7.11). However, approximately 5% of tubules remained shrunken in diameter due to collapse of their epithelium, with irregularly shaped Sertoli cell nuclei and interdigitating areas of Sertoli cell cytoplasm. These tubules often contained only B-type spermatogonia or preleptotene spermatocytes and exhibited a small lumen and peritubular fibrosis(Fig 7.11).

7.4: Discussion:

This study has shown the great potential of testicular cell-specific toxins in the elucidation of cell-cell interactions in the testis. Using a single injection of ethane dimethane-sulphonate(EDS), which has been shown to target exclusively on the Leydig cell and to denude the testis of these cells(Kerr et al, 1985b), it has been possible to study the effects of destruction and regeneration of Leydig cells on the intratesticular distribution of testosterone in relation to changes in testicular morphology. Furthermore the use of short-term screening procedures has identified two other compounds which may repay further study, i.e. methoxyacetic acid and mono-2-ethyl-hexyl phthalate. Although the effects of these latter compounds were not as marked as those of EDS, further study of these compounds could produce greater understanding of the role of germ cell-Sertoli cell interactions in the control of testicular function.

The effects of both MEHP and MAA on testicular function were limited at 2 days post treatment and, in the case of MAA, analysis of results was hampered by small numbers. Both treatments reduced plasma concentrations of testosterone, LH and FSH, although only in the case of FSH was this effect significant($p<0.05$-$p<0.001$, see Fig

7.1). If the fall in plasma testosterone is a result of local impairment of testicular function then it would be expected that plasma LH & FSH would be raised as a result of reduced steroid(and possibly inhibin) feedback from the damaged testis. However, since the results are of a preliminary nature care should be taken before any hard and fast conclusions are drawn. MAA treatment had no effect on the interstitial fluid volume recovered nor on the testis weight of animals within 2 days of treatment. MEHP however significantly increased both IF volume and testis weight, and the latter effect may be a result of the increased fluid content of the testis. Testosterone concentrations in interstitial fluid, seminiferous tubules and in whole testis tissue were also reduced by both MEHP treatment and MAA treatment. However, only with MEHP treatment was the interstitial fluid testosterone content significantly reduced. These results, being of a preliminary nature, are only open to limited interpretation. It is of interest that whilst testicular and plasma testosterone levels are reduced, there is no concomitant increase in plasma LH. Furthermore, despite the presumed presence of tubular damage, as shown previously(Creasy et al, 1983; Foster et al, 1983), plasma FSH levels were also significantly reduced. The effect of MEHP on testis weight and IF volume also suggest further studies would be of value to establish whether these effects persist.

Following treatment with EDS the Leydig cell population was completely destroyed, such that within three days of administration of the toxin no morphologically recognizable Leydig cells were present in any of the testicular sections analysed. This absence of Leydig cells was maintained from 3-7 days post treatment, but thereafter, a new Leydig cell population began to differentiate. These Leydig cells

showed marked differences from normal adult type Leydig cells and closely resembled foetal Leydig cells(see Chapter 1). Only after 28 days did the numbers of Leydig cells increase dramatically, and between 6-10 weeks post treatment these cells were replaced by or transformed into adult-type Leydig cells. However, it is of interest to note that when testosterone levels and Leydig cell numbers within the testis recovered beyond 7 days post-EDS there were striking disparities between the intratesticular testosterone concentration and the numbers of Leydig cells present. When at 2 weeks after EDS administration, the first foetal-like Leydig cells were evident, this correlated with the first detectable levels of testosterone in serum, interstitial fluid, seminiferous tubules and whole testis. However, by 3 weeks interstitial fluid and seminiferous tubule testosterone concentrations had returned to normal, while serum testosterone and whole testis testosterone were only about 50% of control levels(see Figs 7.4 & 7.6). Paradoxically, there were still very few morphologically recognizable Leydig cells within the testis at this time, and whilst detailed morphometric analyses are not yet available, it is highly unlikely that the differences between intratesticular and serum testosterone levels observed between weeks 2-3 could be explained by any increase in Leydig cell numbers seen during this period.

To account for such a dramatic rise in testosterone levels another explanation is required, and there are several possibilities. For example, the high serum levels of LH(approx 500ng/ml) may be responsible for the high testosterone levels, or the small number of Leydig cells present may be hyper-responsive to LH. However, neither of these possibilities would account for the difference in

testosterone levels between 2 and 3 weeks post-EDS(see Fig 7.6). A third possible explanation for the increase in testosterone output at 3 weeks could be the production of local testicular factors which acted to stimulate Leydig cell testosterone production. There are several possible candidates for such a role including testicular 'LHRH'(Sharpe, 1983 for review), a non-LHRH like polypeptide present in rat testicular interstitial fluid(Sharpe & Cooper, 1984) and other less well defined factors(Parvinen et al, 1984; Sharpe & Rommerts, 1984). The potential importance of local regulators of the Leydig cells has been reviewed elsewhere(Sharpe, 1986). Finally, it is possible that the interstitial cell precursors of the foetal-like Leydig cells which repopulate the testis following EDS treatment acquire the ability to produce testosterone before they become morphologically recognizable as Leydig cells, such as occurs in the human testis in which Leydig cell fibroblastic precursors have been shown to respond to hCG stimulation by production of testosterone (Chemes et al, 1985). If this is the case there may be large changes between 2-3 weeks post EDS in the numbers of such Leydig cell stem cells which could then account for the rapid increase in testosterone production at this time. Future studies should help to resolve which of these possibilities is correct.

At 6 hours after EDS administration, serum, interstitial fluid and seminiferous tubule testosterone concentrations fell dramatically but the total testicular concentration of testosterone remained unaltered. Within the testis it is accepted that testosterone is synthesized exclusively by the Leydig cells, and is then secreted into interstitial fluid from whence it is transported or diffuses to the seminiferous tubules and serum. It therefore seems likely that during

the times when the change in testicular content of testosterone was not reflected by a similar change in testosterone levels in interstitial fluid and seminiferous tubules, that the Leydig cells failed to release testosterone. At 6h post EDS, when this phenomenon was first apparent, it is probable that the retention was due to damage to the Leydig cells, as ultrastructurally at this time there is extensive distension and vacuolization of both the smooth endoplasmic reticulum and the Golgi apparatus(Kerr, Bartlett & Donachie, 1985a), organelles which are known to be principally responsible for Leydig cell testosterone secretion(Ewing et al, 1983).

Interstitial fluid bathes the interstitium and the seminiferous tubules and thus provides a medium of communication between these two compartments. Changes in the testicular interstitial fluid volume therefore can play a major role in the modulation of the concentration of testicular factors. It is with testosterone that this change can be most readily observed. For example, if a constant rate of testosterone production by the Leydig cells is assumed, any decrease in interstitial fluid volume will result in an increase in the concentration of testosterone surrounding the tubules. Similarly, if both the rate of production of interstitial fluid and testosterone fall, then the fall in testosterone concentrations will be less marked than if the interstitial fluid content of the testis remained constant. Modulation of the testicular interstitial fluid volume could therefore provide an additional means of maintaining intratesticular concentrations of testosterone as, for example, has been shown for LHRH(Sharpe, 1984). The rate of turnover of interstitial fluid also affects the transport of serum hormones into the testis, and therefore the flow of LH & FSH into the testis is also affected by any change in

the turnover of interstitial fluid. It could therefore be postulated that, in the event of Leydig cell dysfunction, mechanisms controlling testicular interstitial fluid volume would be activated, to conserve testosterone concentrations within the testis. However, since testicular damage also produces changes in capillary permeability(Setchell, 1980), it is not possible in this instance to draw any conclusions from the data presented above relating to interstitial fluid volumes recovered from EDS treated rats, since both mechanisms may be active at the same time. Nevertheless, the fact that interstitial fluid volumes change rapidly following EDS treatment suggests that mechanisms for the control of interstitial fluid turnover may be activated following testicular damage.

Following EDS-induced destruction of the total Leydig cell population testosterone concentrations in serum and within the whole testis were reduced such that at 3 days post-EDS, serum values were less than 50pg/ml, and interstitial fluid levels were less than 2ng/ml. The levels of testosterone found within the testis following EDS treatment were much lower than those reported after 2 days of LH deprivation(See Chapter 6), and also much lower than those seen at 3-4 days after hypophysectomy(Sharpe et al, 1982), when serum testosterone levels fell to 400pg/ml and interstitial fluid testosterone to 46ng/ml. This suggests that even during total gonadotrophin deprivation following hypophysectomy or after treatment with an LH antiserum the Leydig cells retain the ability to secrete small amounts of testosterone, possibly in response to local stimulatory factors. Since this is the case, and because in EDS-treated animals the pituitary remains functionally intact it is possible, perhaps for the first time, to assess the acute effects of selective and total with-

drawal of testosterone on the seminiferous epithelium.

Three days after EDS treatment, testosterone levels were undetectable in both serum and within the testis, and therefore at this time the first signs of testosterone withdrawal should have become apparent. However, only tubules at stages VII-VIII showed any signs of degeneration and this was slight. Even after at least 5 days of testosterone deprivation(i.e. 3-7 days post-EDS), degenerative changes in the seminiferous epithelium were not as extensive as might have been expected and were limited to stages VII-XI. It appears therefore that the seminiferous epithelium is relatively resistant to short-term testosterone withdrawal. Stages VII-VIII appeared to be the most sensitive to testosterone withdrawal and it is these stages which are considered to be classically androgen dependent(Russell et al, 1981). However, the majority of stages of the seminiferous epithelium appeared qualitatively unaffected by the removal of testosterone for 5 days. The progressive pattern and stage specificity of germ cell degeneration in the testis at seven days after hypophysectomy or after blockade of androgen action on the tubules have been described previously(Russell & Clermont, 1977; Russell et al, 1981). Although no hormonal data was presented in the latter studies, evidence gained from experiments on hypophysectomised rats(Sharpe, Cooper & Doogan, 1981) suggests that major but incomplete depletion of testicular testosterone would have occurred in response to hypophysectomy.

Three days after EDS injection testosterone was undetectable within the testis, and at this time the first degenerative changes in primary spermatocytes at stages VII-VIII were seen, suggesting that these cells are the most sensitive to testosterone removal. Seven days after EDS treatment, damage to the seminiferous epithelium appeared to

be more limited than that described by Russell and his colleagues. The presence of numbers of degenerating primary spermatocytes at stage VIII and also of retained sperm heads within the basal areas of the Steroli cell at stage IX were both reported by Russell and Clermont (1977), although the continued presence of such retained sperm heads at stage X was not described by these authors. Furthermore, Russell & Clermont(1977) reported reduced numbers of step 7 and step 19 spermatids at stages VII-VIII, and although these effects were not readily apparent within seven days of EDS treatment, quantitative analysis of the effects of EDS upon the seminiferous epithelium may be necessary to resolve this point. The discrepancy between the latter results and those of Russell and Clermont(1977) may be explained by the fact that the damage seen at 7 days post EDS may only represent the fifth day after testosterone withdrawal as testosterone levels within the first 24 h of EDS treatment were still relatively high. Alternatively, the high levels of FSH present in EDS-treated rats, which are not present in hypophysectomized rats, may have exerted a protective effect on the seminiferous epithelium. The vacuoles seen within the basal areas of the Sertoli cell by 3 days after EDS, and also thereafter(see above), have been described previously in acutely cryptorchid rats(Kerr et al, 1979b). Their presence at stages VII-VIII in the EDS treated testes suggests that these vacoules, thought to represent disruption of the Sertoli cell tight junctions(Kerr et al, 1979), are induced by testosterone deprivation rather than exposure of the testis to high(i.e. abdominal) temperature. However, it remains to be shown whether this represents a primary effect of testosterone deprivation, or is simply a consequence of the disruption of Sertoli cell-germ cell interactions.

From 2-4 weeks after EDS the damage to the seminiferous epithelium became progessively more extensive despite the fact that testosterone levels within the testis had returned to near normal by three weeks. At the latter time, stages XII-II of the spermatogenic cycle appeared normal and by 4 weeks stages VII-VIII had also shown substantial improvement in the numbers and maturity of the germ cells present. However a general recovery accompanied by the loss of the basal vacuoles and basally degenerating primary spermatocytes was not seen until 6 weeks after EDS. The most likely explanation for this observation is that damage sustained earlier in the cycle of the seminiferous epithelium has a 'flow-on' effect downstream, in which with the passage of time, subsequent and later stages of the cycle are seen to be affected. For example, the normal stage XII-II tubules seen three weeks after treatment would be represented by stage VII-VIII tubules by four weeks, and it is of note therefore that this corresponds to the recovery seen at these times.

By 10 weeks after EDS treatment 4 complete cycles of the seminiferous epithelium would have occurred since the return of normal intratesticular testosterone levels 7 weeks earlier. By this time the large majority of tubules had fully recovered and appeared to show complete and normal spermatogenesis. However, a small proportion of tubules(probably less than 5%) failed to recover spermatogenic activity. These may represent the most severely damaged tubules which will either eventually recover, or which may remain atrophied indefinitely. Such focal impairment of spermatogenesis is commonly seen following the recovery of spermatogenesis from disruption(Labrie et al, 1980; Vickery et al, 1983), but its aetiology remains unknown.

Since no degeneration of the seminiferous epithelium occurred

until at least 3 days after EDS injection it is assumed that EDS has no direct effect on the seminiferous epithelium. This is supported by a recent study (Morris, 1985) which showed that multiple injections of EDS in the rat failed to induce chronic spermatogenic damage and failed to prevent restoration of spermatogenesis.

Serum levels of FSH and LH were both significantly increased 3 days after EDS treament, but whilst serum FSH remained elevated until 6 weeks following EDS, serum LH had returned to normal levels by 4 weeks, and even by 3 weeks was already significantly lower than the maximum levels reached. Serum testosterone, although never fully returning to control levels had stabilized within the normal range by 3 weeks post EDS. This data supports the current theory of differential control of LH and FSH secretion, with LH being regulated by testosterone negative feedback, whilst FSH is under the dual control of steroids and testicular inhibin(see Baker et al, 1983 for review). Due to the marked changes seen in FSH during this study, further investigations are necessary to determine the levels of testicular inhibin following EDS treatment and to relate these levels to the other intratesticular changes described.

These studies have shown the potential of specific toxins for the investigation of testicular function and have identified two such chemicals, methoxyacetic acid and mono-2-ethyl hexyl phthalate, for further investigation. Ethane di-methane sulphonate has been shown to specifically target on the Leydig cells and should provide a powerful tool for the future investigation of seminiferous tubule-Leydig cell communication. It is apparent that the testis has the ability to replenish the population of Leydig cells if the resident generation is compromised or removed by toxicological means. The

seminiferous epithelium also appears relatively resistant to damage in the absence of testosterone within the testis either, as a result of hormonal support from raised gonadotrophin levels or because testosterone is not essential to all stages of the seminiferous epithelium.

CHAPTER 8

GENERAL DISCUSSION

The aim of the studies described in this thesis was to investigate communication between seminiferous tubules and Leydig cells within the testis. Since it is established that qualitative and quantitative maintenance of spermatogenesis requires high levels of testosterone and that the testosterone content of the seminiferous tubules tubules may alter in a cyclical fashion(Parvinen, 1982), as does the size of peritubular Leydig cells(Bergh, 1982), it is likely that a significant degree of communication between these tissues occurs *in vivo*. However, methods for the investigation of such interactions *in vitro* were not available at the time that the present studies were commenced.

Recently a number of static systems for the investigation of such interactions have been developed(See Chapters 3-4; Parvinen *et al*, 1984; Syed *et al*, 1985). However, the results obtained with these systems vary between laboratories(see Chapters 3-4) and according to the Leydig cell preparations used. It has been shown that co-culture with seminiferous tubules inhibits testosterone production by unpurified Leydig cells but stimulates testosterone production by purified Leydig cell preparations(Parvinen *et al*, 1984). Furthermore such systems are subject to a number of complicating factors, thus it is recognized that in static incubation systems, either for co-culture of tissues or for incubation of cells in 'conditioned' medium, the composition of the culture medium is in a constant state of flux, due to medium exhaustion, the build up of cellular metabolites and also of possibly toxic factors. Despite these drawbacks such systems have provided convincing evidence that a factor or factors exist which are produced within the seminiferous tubules and are targeted on the Leydig cells. Co-culture of porcine Sertoli and Leydig cells

produced marked changes in Leydig cell morphology and testosterone production when compared with cultures of Leydig cells alone(Tabone et al, 1984). In the rat, seminiferous tubules have been shown to modulate testosterone production by Leydig cells in a stage-dependent manner(Parvinen et al, 1984), although, in the present studies no such effects were evident(Chapters 3-4).

It has been shown that Sertoli cell-conditioned medium stimulates Leydig cell testosterone secretion in the rat(Grotjan & Heindel, 1982), and recent studies have shown that the apparent levels of a comparable Sertoli cell factor are increased by both FSH- and dibutyryl cAMP-stimulation of the Sertoli cell cultures(Verhoeven & Cailleau, 1985). However, Syed et al(1985) have shown that seminiferous tubule-conditioned medium produces inhibitory effects on testosterone production by unpurified Leydig cells. Such results agree with those of Parvinen et al(1984) obtained with a co-culture system using unpurified Leydig cells. The system described in the present study demonstrated stimulatory effects of seminiferous tubule-conditioned medium on testosterone production by Percoll-purified Leydig cells(Chapter 4). However, such effects were highly variable and initial results of studies in which the active factor(s) were concentrated by ammonium sulphate precipitation were inconclusive. Nevertheless, it is apparent from these studies and those in the literature, that more than one factor is produced by the seminiferous tubules which can affect Leydig cell testosterone production. Such factors, presumed to be of Sertoli cell origin, include; 'testicular LHRH'(Sharpe, 1982 for review), an interstitial fluid factor or factors(Sharpe & Cooper, 1984) and possibly factors present in seminiferous tubule-conditioned media(see above and Chapter 4). It

remains to be firmly established whether the activities of any of these factors are of physiological importance, and whether any of them represent identical factors.

In the light of the variability of the results produced by static culture systems and also their drawbacks a system for the study of dynamic interactions between seminiferous tubules and Leydig cells was investigated. Previous studies on the perfused testis have proved of some value in the elucidation of testosterone biosynthetic pathways in an *in vitro* system(Chubb & Ewing, 1979a-c). However, such a system has severe limitations when used for the study of interactions between the seminiferous tubules and Leydig cells, because it is not possible to study the individual components of the testis in such a system. A perifusion system based on a system previously validated for the perifusion of unpurified Leydig cells(Wu *et al*, 1985) was adapted and validated for co-perifusion of Leydig cells with seminiferous tubules. Such a system enables the investigation of direct communication between these tissues in a dynamic state. Using this system it has been possible to demonstrate that at a ratio of Leydig cells to seminiferous tubules similar to that seen *in vivo*(i.e. approx. 3 million Leydig cells/200 cm seminiferous tubules) Leydig cell testosterone production was significantly increased by the presence of seminiferous tubules, and that, at 'non-physiological' ratios(i.e. 3 million Leydig cells/50 cm seminiferous tubules) Leydig cell testosterone production was decreased(See Chapter 5). Whilst the latter finding is still of a preliminary nature, it may help to explain the inhibitory effects on unpurified Leydig cells of seminiferous tubules and seminiferous tubule-conditioned medium reported in other studies(Syed *et al*, 1985; Parvinen *et al*, 1984). The

latter findings may be explained by postulating that either the ratio of Leydig cells to seminiferous tubules was unphysiological, or that other cell types, particularly germ cells and macrophages, present in the un-purified cell preparations interact with the Leydig cells to further affect their testosterone output. Presumably any effects of germ cells on the Leydig cells would be aspecific, as it is difficult to conceive how germ cells could directly communicate with the Leydig cells in a physiological situation.

Whilst it has yet to be proven that macrophages have a paracrine role within the testis, recent studies suggest that this may be the case. In macrophages FSH has been shown to increase amino acid and uridine incorporation into acid precipitable material *in vivo* and *in vitro*(Yee & Hutson, 1985a,b) and these cells have been shown to possess specific FSH receptors(Yee & Hutson, 1985a). Furthermore, macrophage-conditioned culture medium has been shown to affect Leydig cell testosterone production and the levels of stimulatory factors in macrophage-conditioned medium were increased in a dose-related fashion by FSH(Yee & Hutson, 1985c). Macrophages make up approximately 10-15% of the cells in the Percoll-purified Leydig cell preparations used in the present studies, and *in vivo* the ratio of Leydig cells to macrophages is about 4:1(Bergh, 1985). Macrophages have been shown to be closely associated with Leydig cells(Miller *et al*, 1983; see also Chapter 1), and whilst the possibility that macrophages play a role in the paracrine regulation of Leydig cells has not previously been widely considered, evidence is growing that such a role may exist. In this light, the ability of EDS to specifically destroy Leydig cells *in vivo*(Chapter 7), may enable the preparation of macrophage-enriched, Leydig cell-free preparations which should permit

further studies in this area to be carried out.

Some degree of 2-way communication between Leydig cells and seminiferous tubules in vitro may be demonstrable using the perifusion system, since the effect of seminiferous tubules on testosterone production by Leydig cells has been shown to vary according to the LH dose to which these cells are exposed(Chapter 5). However, the possible involvement of macrophages in this context has not yet been considered and the use of macrophage-enriched preparations may be of value in this situation.

The validation of a perifusion system for the investigation of dynamic interactions between seminiferous tubules and Leydig cells, in the absence of such problems as build up of toxic wastes and end-products, should provide a powerful tool for further studies. Results suggest that such a system will enable investigations to proceed along two initial avenues: Firstly, to use the system as an in vitro bioassay for the identification of possible paracrine factors, such as the endorphins, and their effects on perifused Leydig cells or on columns containing both Leydig cells and seminiferous tubules. Secondly, to characterize the effects of such factors and to elucidate the hormonal control of interactions between seminiferous tubules and Leydig cells. For example, the effects of either 'pulsing' the system with FSH or β-endorphin or the addition of these hormones to the perifusion system for longer periods(1-6 h) could be used to study their effects. Further purification of the factor(s) present in seminiferous tubule-conditioned medium or interstitial fluid, and subsequent perifusion of Leydig cells with either the isolated or partially-purified factors may also provide further information about the actions of these factors. The effects of toxins targeted on

specific cell types either *in vitro*, using for example MEHP(Chapter 7) to impair Sertoli-germ cell interactions, or *in vivo*, using EDS to produce testosterone-deprived tubules may also be used to investigate the importance of both cellular interactions and cellular function. Finally, the use of various *in vivo* treatments in rats, or the use of human interstitial cells or tubules may also provide information about the paracrine regulation of the testis. At present, the perifusion system developed herein provides possibly the best opportunity for the study of intratesticular paracrine regulators of Leydig cell function, and its further application should yield much interesting and novel information about this regulation.

Methods for the quantification of testosterone concentrations within different compartments have also been established and validated (Chapter 6). Testicular testosterone levels were reduced either by removing peripheral LH using a potent antiserum or by inducing experimental cryptorchidism(Chapter 6). In early cryptorchidism, reduced testosterone levels *in vivo* are observed, whilst *in vitro* Leydig cells are hyper-responsive to LH(Sharpe, Doogan & Cooper, 1981). This apparent paradox may be a result of a sharp reduction in testicular blood flow during cryptorchidism, which could result in a reduction in the levels of LH reaching the Leydig cells in the cryptorchid testis, since serum LH levels do not alter during short term cryptorchidism(Chapter 6). Whilst this hypothesis is attractive, it is not proven. In both cryptorchidism and following anti-LH treatment, there is evidence that levels of a testicular factor(s) which stimulates Leydig cell testosterone production are increased(Sharpe *et al*, 1985). Despite this change, intratesticular

testosterone levels are markedly reduced shortly after withdrawal of LH stimulation. Also, during the co-perifusion of seminiferous tubules with Leydig cells or the culture of Leydig cells with interstitial fluid the most marked stimulation of Leydig cell testosterone production was seen in the presence of LH or hCG stimulation(Sharpe & Cooper, 1984; Chapter 5). This data may be explained by suggesting that the role of such local factors is to interact with and supplement peripheral LH stimulation rather than to replace it. If this is true then it would appear that the function of paracrine regulators in vivo is to respond in situations in which LH stimulation is insufficient to maintain intratesticular testosterone levels, thus providing a mechanism for the control of testosterone levels within the testis. However, this interpretation is largely speculative at present.

A further mechanism for the maintenance of spermatogenesis could be by the retention of testosterone within the seminiferous tubules. Following treatment with an antiserum to LH the ratio of testosterone in the seminiferous tubules to that in the surrounding interstitial fluid rose significantly(Fig 6.6). Furthermore the regression line representing the relationship between seminiferous tubule and interstitial fluid testosterone for both cryptorchid animals and those treated with anti-LH was not representative of a 1:1 relationship, again suggesting that testosterone may be preferentially retained within the tubules. However, following EDS treatment, which destroys Leydig cells and abolishes testicular testosterone(Chapter 7), the same relationship was not evident and the testosterone concentration in both seminiferous tubules and interstitial fluid declined at the same rate. Therefore, it would appear

likely that for testosterone to be preferentially held within the seminiferous tubules at times when testicular testosterone content is low, the presence of functionally competent Leydig cells is required. If this is true it would imply that rather than the seminiferous tubules being able to store testosterone or to actively concentrate testosterone, the Leydig cells preferentially direct testosterone into the seminiferous tubules during testosterone deprivation. Whilst it is unlikely that Leydig cells secrete testosterone uni-directionally, the presence of a peritubular population of Leydig cells may result in the formation of a locally high testosterone concentration proximal to the seminiferous tubules. Furthermore, such cells, being positioned directly adjacent to the seminiferous tubules might also be exposed to higher concentrations of paracrine factors derived from the seminiferous tubules. Since peritubular Leydig cells adjacent to stages VII-VIII of the spermatogenic cycle have been shown to be larger than those situated perivascularly(Bergh, 1982) such a relationship may exist _in vivo_. However, whilst such an arrangement is attractive to postulate, its existence is speculative and will prove difficult to test in practice.

The availability of techniques for the investigation of the distribution of testosterone will also allow studies on the control of testosterone utilization within the testis to be carried out. For example, it has been suggested that FSH increases seminiferous tubule testosterone uptake _in vivo_(Seilicovich & Rosner, 1972), and treatment of animals with FSH may provide further information on this putative action of FSH. These techniques may also be used in conjunction with the _in vitro_ perifusion system

described above, to assess the in vivo effects of any putative paracrine regulatory factors on interactions between seminiferous tubule and Leydig cells as reflected by both the intratesticular distribution of testosterone and the levels of an interstitial fluid factor(s) which acts on the Leydig cells(Sharpe & Cooper, 1984).

These studies have also shown that chemical toxins should prove extremely useful tools for the investigation of testicular function (Chapter 7). The use of a specific Leydig cell toxin, ethane-dimethane sulphonate(EDS), has provided a novel and powerful tool for the investigation of testicular function. Previously, studies on the in vivo role of testosterone have been hampered by the ability of Leydig cells, in the absence of any pituitary stimulation, to produce appreciable although markedly reduced quantities of testosterone(see above and Sharpe, Cooper & Doogan, 1981). However, by specifically removing the Leydig cell population from the testis it has been possible to study the effects of complete testosterone withdrawal on the spermatogenic process without removing stimulation of the testis by peripheral hormones. By monitoring the morphological changes in both the interstitium and the seminiferous tubules following EDS treatment, it has been possible to state with reasonable certainty, that this chemical does not, in the rat, affect the seminiferous tubules directly, but that its effects are centred on the Leydig cells. It can therefore be assumed that the effects on the seminiferous tubules described in this study(see Chapter 7) are due wholly to the removal of the Leydig cell population, and probably to the resultant decrease in testicular testosterone concentration. Therefore it is of interest to note that, following removal of testicular testosterone, damage to the seminiferous epithelium was

less extensive than expected and was initially restricted to stages VII-VIII of the spermatogenic cycle. By seven days post EDS, which represents 5 days post testosterone withdrawal, the incidence of damage increased to cover stages VII-XII. It would appear that stages VII-VIII are the most sensitive to testosterone(Chapter 7; Russell et al, 1981), but the fact that most stages of the spermatogenic cycle were unaffected by removal of testosterone strongly suggests that many of the stages of the cycle are not dependent on testosterone. However, it should be noted that these conclusions are based on qualitative rather than quantitative observations and studies on the quantitative effects of testosterone withdrawal are needed to provide a clearer picture.

From these studies it has been possible to identify the primary spermatocytes at stages VII-VIII as the cells most sensitive to testosterone withdrawal. The germ cell damage seen following EDS-induced Leydig cell destruction was less extensive than that observed following hypophysectomy(Russell & Clermont, 1977; Russell et al, 1981) possibly due to increased FSH levels exerting a protective effect on the spermatogenic process.

However, before such studies are attempted the specificity of such toxins must be clearly established, involving detailed and time-consuming evaluation of testicular morphology. In this study a specific Leydig cell toxin(EDS) has been described with preliminary findings relating to two further toxins which act, respectively, on Sertoli cells and on a specific generation of germ cells. Whilst studies on EDS have been limited to the in vivo situation the potential of this compound and others for the study of the effects of "damaged" seminiferous tubules on Leydig cells in vitro using the

perifusion system is considerable: e.g. it should be possible to compare the effects of normal untreated seminiferous tubules with tubules lacking a specific germ cell generation and tubules deprived in vivo of normal testosterone supplies, and to use these to study interactions between seminiferous tubules and Leydig cells.

EDS treated animals have also shown the ability to replenish the testicular Leydig cell population. In the human, the Leydig cell precursors appear able to produce testosterone prior to acquiring the morphological characteristics of Leydig cells(see Chapter 1). As may be suggested by the evidence shown above(Chapter 7), this also occurs in the rat, and assuming that the normal Leydig cell population of the testis is continually turning over, the presence of such precursor cells in the normal testis may further complicate the study of the paracrine regulation of the testis by providing a hitherto unrecognised or unconsidered population of steroidogenic cells.

The use of specific toxins will prove of great value in the unravelling of cell-cell relationships within the testis, and especially within the seminiferous tubule. This could have possible long-term benefits related to our understanding of the causes of idiopathic infertility in men, especially those cases(the majority) clearly involving abberations of spermatogenic development. Furthermore, such studies will serve to highlight potentially damaging chemicals in the environment, from which category, two of the compounds used in these studies were drawn. Mono-ethyl hexyl phthalate (MEHP) is derived from one of a class of plasticizers used throughout the plastics industry to which normal males are continually exposed(through the use of plastic containers for food and drink and also the use of plastics for storing blood for transfusions), whilst

methoxy acetic acid(MAA) is a metabolic derivative of a compound found in most paints. Both of these compounds are potentially valuable tools for the study of seminiferous tubule cell-cell interactions, since MEHP appears to act specifically on the Sertoli cell to cause exfoliation of germ cells, whilst MAA appears to act selectively on primary spermatocytes undergoing post-zygotene meiotic maturation and division(see Chapter 7). Studies similar to those carried out on EDS and possibly involving perifusion of treated seminiferous tubules should provide useful information relating to the importance of cellular interactions within the tubules for the maintenance of testicular function and spermatogenesis.

In conclusion, these studies have established novel and powerful techniques for the investigation of the paracrine regulation of testicular function. The perifusion system described represents a significant improvement upon the in vitro systems previously available for the investigation of seminiferous tubule-Leydig cell interactions. Results obtained with this system suggest that communication between these cell types may act in both directions, as might be expected for a physiological feedback regulation system(Chapter 5). Techniques for the determination of intratesticular distribution of testosterone, when combined with histological and toxicological techniques, have provided insights into the role of testosterone in the regulation of spermatogenesis and have also identified chemicals which may further advance our understanding of the role of intratubular cell associations in the maintenance of testicular function. It is apparent from these and other studies that seminiferous tubule-Leydig cell interaction, far from being a 'simple' dynamic system involving only Leydig and Sertoli

cells, also involves germ cells, and possibly macrophages and Leydig cell precursors(Chapter 7), and it seems certain that many paracrine factors will be involved in such communication.

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